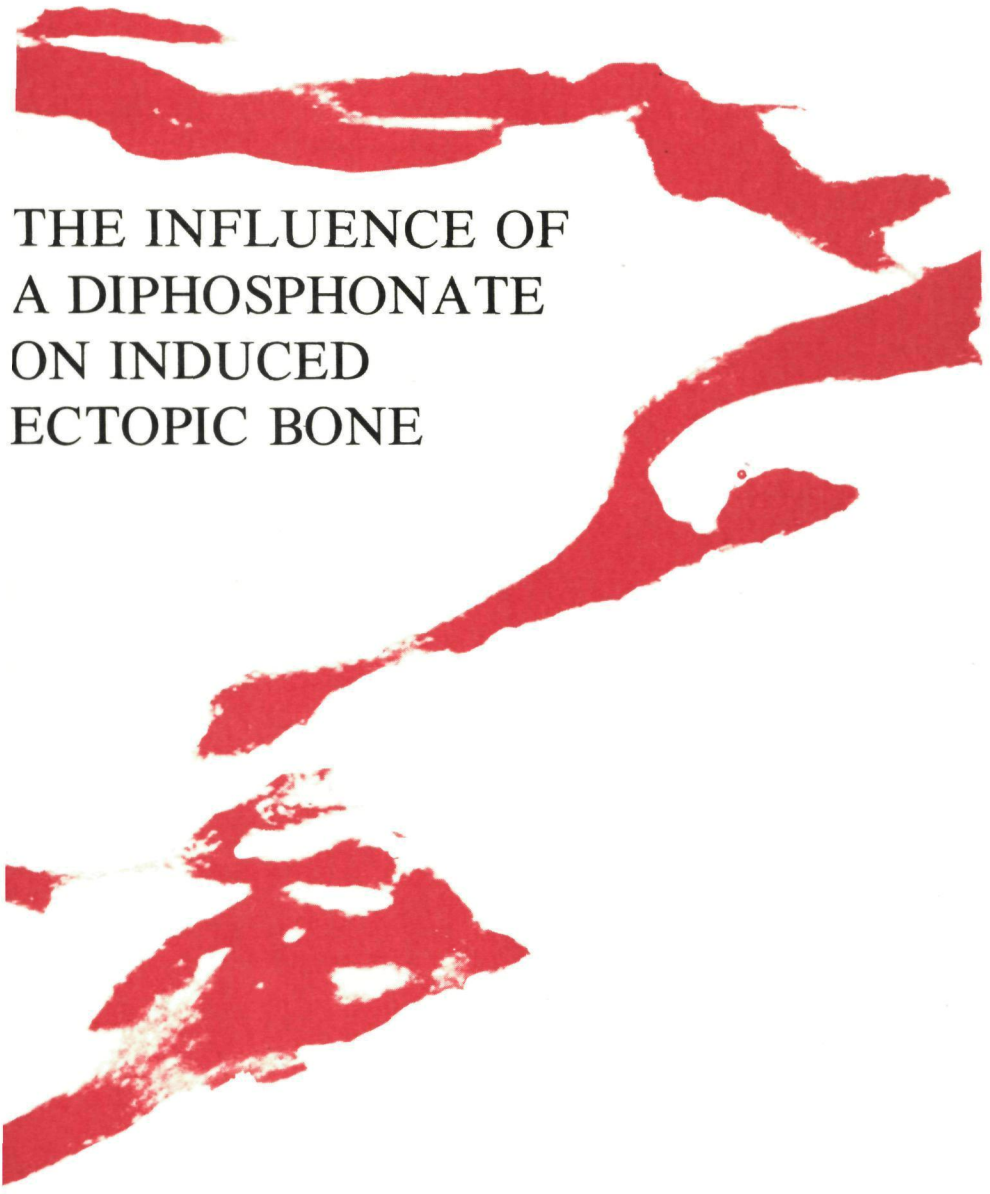

THE INFLUENCE OF A DIPHOSPHONATE ON INDUCED ECTOPIC BONE

CHR. PLASMANS

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THE INFLUENCE OF A DIPHOSPHONATE ON INDUCED ECTOPIC BONE

A histological, fluorescence-microscopic and electron-microscopic study on induced ectopic bone in the rabbit.

Promotor:	Dr. T. J. J. H. Slooff
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A histological, fluorescence-microscopic and electron-microscopic study on induced ectopic bone in the rabbit.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. A. J. H. VENDRIK, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 29 APRIL 1977 DES NAMIDDAGS TE 3.00 UUR PRECIES.

door

CHRISTIAAN, MICHAËL, THOMAS, JOHAN, MARIE PLASMANS

geboren te Oss

1977
Drukkerij Van Mameren, Nijmegen

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Aan Ellie
Aan mijn ouders

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CONTENTS

The motivation of this study originates from clinical facts. With the increasing number of total hip replacements in orthopaedic surgery, periarticular heterotopic ossifications are being increasingly identified as a complication of this procedure. In many cases this complication partly abolishes the functional capacity which results from the operation.
The etiological factors underlying these ectopic ossifications have so far remained obscure. It might be suggested that a surgical procedure and/or the implantation of a foreign body can induce periarticularly localized mesenchymal cells, thus creating a cell system which can result in the formation of heterotopic bone.
A trial in our clinic has demonstrated that diphosphonates can be succesfully used in the prevention of these periarticular heterotopic ossifications, and ultimately safeguard increased and less painful mobility. Diphosphonates belong to the group of polyphosphates, which also includes pyrophosphate. Pyrophosphate, which normally occurs in biological fluids, is submitted to rapid hydrolysis in vivo. In consequence of this property, it is impossible to use this compound for therapeutic and experimental purposes. Therefore the clinical used ethane-1-hydroxy-1,1-diphosphonate (EHDP), which is not hydrolysed in vivo, was selected.
It is assumed that, apart from its extracellular activity in the form of direct inhibition of the growth and degradation of apatite crystals, pyrophosphate also interferes with the metabolism of bone cells. EHDP is known to exert this extracellular effect, but a possible effect on the cellular metabolism is uncertain.
The influence of EHDP on an induced cell system was studied. We chose for the induction-model in the rabbit documented by Urist and his co-workers, which made it possible to analyse events in bone induction at certain intervals after a known starting point (T_0), with a high degree of reproducibility. In this set-up it is likewise possible to study, at the same intervals, the influence of administration of EHDP on the induced cell system. The decision to make use of a heterotopic induced cell system was partly determined by the desire to avoid a possible influence of existing bone on the mechanism of induction.
In order to obtain some information on the cellular effect of EHDP, EM-techniques were used in a limited number of experiments.
The first part of this thesis comprises a survey of the literature on ossification, bone induction and diphosphonates.

The second part deals with the methods used, the results obtained, followed by the discussion of the results.

BONE; A DYNAMIC TISSUE

Our test arrangement was subservient to the study of heterotopic ossifications and the influence of the diphosphonate ethane-1-hydroxy-1,1-diphosphonate (EHDP) on these ossifications. For interpretation of the results it is necessary to be informed of modern views on the mineralization process. Although a study of recent literature and reviews shows that many uncertainties still exist, this chapter is nevertheless intended to outline the most widely accepted views

Function of bone tissue

Macroscopic and microscopic changes observed in bone tissue in vivo, can be regarded as reflecting the functions (or dysfunctions) of the tissue. Bone tissue serves two functions.

1) Skeletal modelling and remodelling, which ensures the growth and homeostasis of the supporting apparatus During growth but also at an adult age, bone tissue is being constantly broken down and newly formed. Beside the important genetic and hormonal control, tensile and compression forces are also involved in this process In the latter case the piezoelectric properties of the bone mineral play an important role (Bassett 1968; Becker & Murray 1967; Currey 1970).
2) After mineralization, the newly formed bone has a function in the mineral homeostasis. Bone cells have an important contribution to regulate the concentrations of minerals in the intracellular and extracellular fluid.

Composition of bone tissue

The structure of a long bone is unmistakably more compact at the surface than at the centre. The dense structures consist of long, slender cylinders arranged along the longitudinal axis of the bone The centre of these cylinders is occupied by a blood vessel, and in the 17th century Havers described these as structural units (Bonucci & Ascenzi 1970) These structural units (Haversian systems or osteons) have a spiral shape in relation to their own longitudinal axis, adjacent osteons, and to the longitudinal axis of the skeletal part. Beside the degree of mineralization, this spatial arrangement is one of the

determinants of the mechanical properties of bone tissue (Cohen & Harris 1958; Vincentelli & Evans 1971).
Closer examination shows that the structures in the centre of the long bone consist of thin trabeculae, arranged in a certain pattern which is reminiscent of a rather imperfect honeycomb. Bone with this structure is called spongy bone. The compact and spongy bone tissues are continuous with each other. Both consist of bone cells and extracellular matrix, but the main difference relates to the degree of vascularization and the arrangement of the bone cells in reference to the vascular ramifications. The compact bone is permeated by numerous blood vessels. Spongy bone on the other hand is composed of delicate bone trabeculae and spiculae with irregularly dispersed bone cells. The blood vessels are largely confined to the hemopoietic tissue which fills the cavernous spaces between the trabeculae.
Different types of bone are formed at different ages, woven (or fibrous) and lamellar bone. Lamellar bone is formed only in apposition to an existing surface. Each osteoblast is coordinated with his neighbour and together they make a continuous layer of bone on one side only. Woven bone is laid down directly in condensations of fibrous tissue by the action of individual osteoblasts without the necessity for the presence of an adjacent free bone surface. Each osteoblast surrounds itself on all sides with an islet of new matrix without relation to its neighbour. In woven bone mineralization occurs rapidly and diffusely in relation to the collagen fibers, in contrast to the regular grain of lamellar bone. Osteocytes are much more frequent, and are scattered at random with no relation to vascular channels. All bone formed in locations where previously no bone existed is initially woven bone. The formation of woven bone is followed by its piecemeal resorption and gradual replacement by lamellar bone. Bone comprises, in addition to cellular constituents, an organic and inorganic tissue component. In this section a morphological and functional description of the various types of bone cells will be given. In addition the organic and inorganic components and their function will be discussed. First of all, however, the origin of the bone cells should be discussed.

The origin of the bone cells

As indicated in fig. 1, it is generally assumed that bone cells arise from not-yet-differentiated mesenchymal cells localized in the perivascular connective tissue (Remagen 1973; Vaughan 1970; Vittali 1970; Young 1962; Young 1963).
The functions of the bone tissue cells are regulated by genes localized in the cell nucleus. Only a few genes are active and the remaining genes are reversibly blocked, thus determining a given bone cell function. This gene

activity is subject to changes caused by stimuli which affect the cell. In this concept, therefore, there are not so much different cell types as different states of cellular function. This reversible functional change is known as modulation. Extraneous stimuli can also cause cell differentiation. This implies maturation of the cell from a low to a high level of specialization – again a reversible process.

It is useful to mention a different bone cell model, in which every osteoblast is thought to be formed by differentiation from an osteoclast (Jee et al. 1972; Meunier et al. 1971; Rasmussen & Bordier 1974a).

With these views in mind, the various cell 'types' can now be discussed.

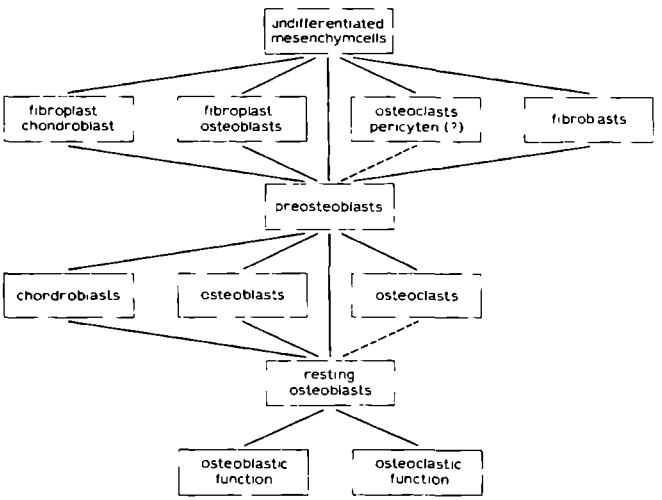


fig. 1. Diagram of the origin of bone cells (According to H. P. Vittali 1970).

Osteoblast

The osteoblast is readily identifiable at light microscopy. It is of cubical or cylindrical shape, has a large nucleus, a juxtanuclear halo and basophilic cytoplasm. At electron-microscopic examination the halo proves to correspond with a well-developed Golgi apparatus, in which high-molecular carbohydrates such as mucopolysaccharides are present. The basophilia results from the presence of an abundance of rough endoplasmic reticulum – a reflection of the high protein synthesis. The osteoblast has cytoplasmic processes connecting it with adjacent osteocytes (Cameron 1972; Remagen 1973; Schenk 1974).

Because of their close proximity to each other, they appear to form a barrier between the surface of the forming bone on one side and the connective tissue

and blood vessels of the marrow on the other side.
The function of the osteoblast is a complex one. Osteoblasts form osteoid, a tissue, which is characterized by the ability to be mineralized. After termination of this activity the osteoblast is either 'incorporated' in the osteoid which is in process of mineralization, remains behind on the bone surface as a surface osteocyte or resumes function as a preosteoblast (Remagen 1973). It is possible that the osteoblast has also an important function in initiating the mineralization. Osteoblasts may have the ability to accumulate calcium and phosphate in the mitochondria. An attractive theory is that these minerals are transported to the cell periphery, packed in vesicles, and then extruded into the extracellular space. Several authors are stressing that these extracellular concentrations of calcium and phosphate ions can serve as nucleation centres in osteoid mineralization (Brighton & Hunt 1976; Matthews 1970; Matthews & Martin 1971; Nichols & Rogers 1971; Schenk 1974; Shapiro 1971; Wadkins et al. 1974). Calcifying extracellular matrix vesicles have been identified by electron microscopy in cartilage, bone and dentine (Anderson 1973). Chemical analysis showed that these vesicles contained high concentrations of calcium phosphate compounds, sometimes in a crystalline form. The calcium phosphate compounds are released as a result of enzymatic degradation or perforation of the vesicle membrane, and can then function as nucleation centres (Ali 1976; Anderson & Sajdera 1976). In some publications it is suggested that the extracellular vesicles described by Anderson are the original cellular vesicles, but a definitive proof is not yet presented (Bernard & Pease 1969; Kashiwa & Komorous 1971; Rabinovitch & Anderson 1976; Slavkin et al. 1976; Wuthier 1976). The above described mineralization activity is associated with a high concentration of alkaline phosphatase activity in the mitochondria as well as in and outside the extracellular vesicles (Anderson 1973; Arsenis 1972; Salomon 1974).

Osteoclast

Bone is resorbed as a whole, both mineral and matrix, by osteoclasts which are multinucleated giant cells, short lived, mobile and closely apposed to the bone surface. The area in contact with the bone is ruffled and irregular in the center with a peripheral clear zone which is free of cellular organelles. The ruffled border has numerous cytoplasmic extensions infiltrating the desintegrating bone surface. Variations in the activity of the cell may be accomplished by varying the proportion of ruffled border to clear zone; some osteoclasts may have no ruffled border at all and are presumed to be, at least temporarily, inactive. In the cytoplasm, many mitochondria and vacuoles are present beside many denser, vesicular structures: the so-called lysosomes. In these structures the enzymes characteristic of lysosomes, which are also

involved in intracellular digestion of phagocytized bone remnants, were demonstrated (Rasmussen & Bordier 1974b, Remagen 1973, Schenk 1974; Walker 1972). These lysosomes are formed in the very highly developed Golgi apparatus (Scott 1967) Vaes and his co-workers maintain, that in biochemical terms, an acid milieu and the presence of extracellular lysosomal enzymes are prerequisits for the resorption (Vaes 1971; Vaes & Vreven 1971).

Although mesenchymal cell proliferation, osteoclastic resorption, and osteoblastic formation follow one another in unvarying succession, the nature of the coupling between these processes is still obscure. Many types of connective tissue cells in bone, in bone marrow and outside the skelet have the capacity to form osteoclasts. Under the right condition, these may include macrophages and monocytes (Hall 1975). Gothlin and Ericsson (1973) were able to demonstrate in parabiotic rats that osteoclasts in callus tissue are derived from blood-born macrophages and monocytes. However, osteoclasts could also probably locally arise by the fusion of cells derived from the proliferation of a specific mesenchymal cell type, the so-called osteoprogenitor cell (Vittali 1970). An alternative possibility is that existing mesenchymal cells can fuse into osteoclasts without prior cell division (Horn et al. 1975)

Osteocyte and surface osteocyte

Light-microscopically, the active osteocyte is characterized by a highly basophilic cytoplasm Electron-microscopically, this is reflected in the amount of rough endoplasmic reticulum and the level of free polysomes (Bonucci et al. 1969, Cameron 1972; Schenk 1974).

The bone surface is covered by a layer of thin, flattened cells which form an incomplete and leaky envelope This layer of surface osteocytes is found lining the Haversian canals as well as the endosteal and trabecular surface. Because of their anatomical and functional relationship with the deep osteocytes the term 'surface osteocyte' is adopted.

Approximately one osteoblast in ten becomes buried in the bone as an osteocyte, each lying in its own lacuna. These cells remain in communication with each other and with the cells on the surface by means of protoplasmic processes lying within canaliculi. Between the plasma membrane of the osteocyte and the lacunar and canalicular wall is a thin sheath or capsule of amorphous material, containing few or no collagen fibers and a large amount of mucopolysaccharides through which water and ions can diffuse (Remagen 1973). Some observers believe that movement of water and ions is accomplished in some way by microtubules within the cytoplasm of the cell and by microfilaments within the cell processes (Weinger & Holtrop 1974)

For decades, the osteocyte has been regarded as an inactive cell buried in the bone. But according to modern concepts these cells play an active role. A thin layer of bone, immediately adjacent to the wall of the osteocyte is usually referred to as perilacunar bone It differs from bone elsewhere (interlacunar bone) in several respects. The collagen fibers are fewer, more loosely packed, and less densely mineralized and the mineral is more soluble, more amorphous and less highly crystallized (Mjor 1962). It is generally accepted that the bone immediately adjacent to the osteocyte is metabolically active and subject to cyclical removal and replacement (Parfitt 1976a) A process which has been termed 'osteocytic miniremodelling'. The close juxtaposition of periosteocytic resorption within and osteoclastic resorption on the surface of the bone, suggests that these two types of cells are working together as a functional unit, engaged in endosteal resorption The mechanism of this local coupling is unknown. Beside this remodelling function there is an active role in calcium homeostasis. A temporary demineralization of the metabolically active perilacunar bone as a component of this homeostasis, is possible It functions as a rapid response to errors in the prevailing steady-state level of plasma calcium. It is a phenomenon called 'osteocytic osteolysis'. (Baud & Auil 1971, Belanger 1969, Bordier & Tun-Chot 1972)

Calcium moves in both directions between plasma and bone. It is known that this exchange of calcium is in some way involved in plasma calcium regulation. According to modern concepts it is possible to design a highly schematic bone model with several fluid compartments which are separated by cellular barriers. The fluid between the surface osteocytes and the bone and between the deep osteocyte and its processes and the canalicular and lacunar walls is assumed to have an ionized calcium concentration of about one third that of plasma (Doty & Schofield 1972; Neuman & Ramp 1971; Matthews 1970) (fig. 2).

The inward flux depends on the concentration gradient, calcium ions flowing out of the ECF (extracellular fluid) in the ground substance of the connective tissue lying between the blood vessels and the bone and through the gaps between the surface osteocytes toward the bone fluid. The outward flux is dependent on calcium ions pumped out of the surface osteocytes back toward the connective tissue ECF, which is presumed to have a Ca^{++}-composition similar to that of ECF elsewhere and to be in equilibrium with the plasma (Talmage 1969). The calcium ions pumped out by the surface osteocytes are derived from calcium ions which flow into these cells through their inner walls facing the bone and facing the intercellular pores, or which move from deeper regions of bone toward the surface. The nature of the pathway between deep and surface osteocytes is unknown.

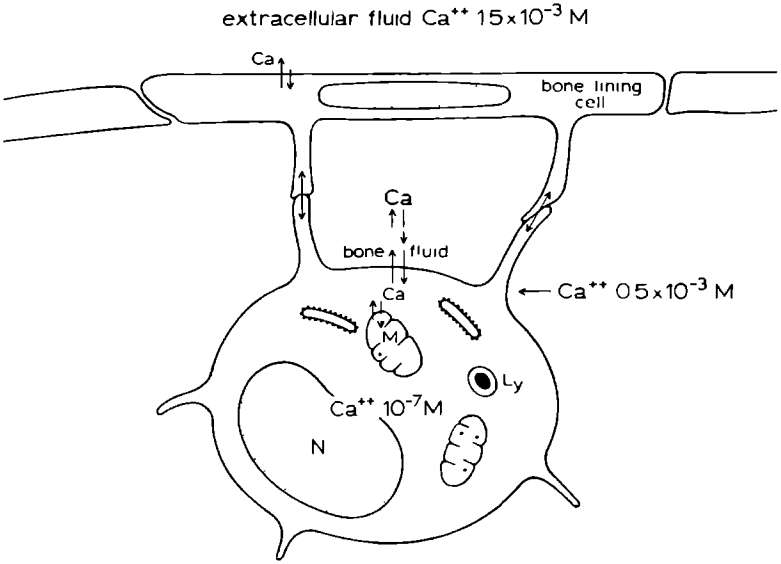


Fig 2 Diagram of the relationship between the osteocyte and the bone lining cell (surface osteocyte) in calcium-transport N=nucleus, M=mitochondria and Ly=lysosome

Organic and inorganic bone constituents

This section discusses the other bone constituents, including some of their properties as are relevant to the mineralization process. Freshly obtained bone consists of about 35% organic material and water, and about 65% minerals (Robinson 1975). When bone is decalcified by means of acid, the bone matrix of organic material remains. As soon as the groundsubstance, formed by active bone cells, is ready to be mineralized, it is referred to as osteoid. Collagen is a very important constituent of the matrix, and comprises some 95% of the organic material (Robinson 1975)

So far, no satisfactory explanation has been offered for the observation that the bone matrix, unlike comparable matrices in other tissues, is normally mineralized in vivo. The explanation was sought in what was believed to be the unique composition and structure of bone collagen.

Dehm and his co-workers established that, in the osteoblast, polypeptides are formed from amino-acids (e g glycine, proline) and then transferred to the extracellular space. Tropocollagen – the ground substance of collagen – is made up of three of these polypeptide chains, which jointly spiral about a common axis (Dehm & Prockop 1971;Dehm et al. 1972; Grant & Prockop

1972). At electron-microscopic examination, the collagen fibrillae show a 'cross striation' with a periodicity of 64 nm. In an effort to explain this phenomenon, Petruska & Hodge (1964) introduced the model of 'quarter staggering'. In this model the molecules are arranged with their longitudinal axes parallel. Two consecutive longitudinally arranged molecules are separated by a space of 37.5 nm. Two molecules arranged side by side have been so staggered that a new molecule appears every 64 nm. Several authors postulate an important role of bone collagen in mineralization. In view of the special stereometric configuration indicated in the model of Petruska & Hodge, bone collagen could be a nucleation catalyser. These authors base their views on observations indicating that only collagen with the 64 nm-band can be mineralized, and that each nucleation should begin in 'holes' localized at this latitude (Bachra 1970; Cobb et al. 1976; Glimcher & Krane 1968; Cameron 1972; Eastoe 1970; Wadkins et al. 1974).

Besides this possibility recent publications have been written describing anionic proteins co-valently bound to osseous and dental collagen, which are believed to be capable of binding phosphorus. According to Shuttleworth & Veis (1972) and Spector & Glimcher (1972), these anionic proteins could play an important role in starting mineralization.

The association of the start of each nucleation to collagen, however, is disputable. Electron-microscopic studies have clearly shown the presence of nucleation centres unrelated to bone collagen (Bernard & Pease 1969; Boothroyd 1975; Walton 1974).

The collagen fibrillae are embedded in a kind of binding substance. An important organic constituent of this substance consists of acid mucopolysaccharides such as chondroitin sulphate. Is has long been thought that acid mucopolysaccharides play an important role in the mineralization process. In the early Fifties it was postulated by Sobel & Burger (1954) that these compounds function as nucleation sites. On the other hand several investigators regard them as inhibitors of mineralization because the anionic groups in the acid mucopolysaccharides can trap and bind the calcium from the milieu (Baylink et al. 1972; Campo et al. 1969; Pita et al. 1970). More recently, Kuettner et al. (1975) described a lysosomal disintegration of acid mucopolysaccharides which abolishes their inhibitory effect and in addition ensures the release of an abundance of calcium for mineralization.

Studies on collagen as a nucleation catalyst for hydroxyapatite formation have received much attention for years. Although it was demonstrated by Irving & Wuthier (1968) that lipids can bind calcium, yet a possible function of the lipids in mineralization has for years remained obscure (Irving 1973). It is now becoming apparent that specific acidic lipids are also closely associated with the mechanism and that the loci for initial mineral deposition could

be the extracellular matrix vesicles (Anderson 1973, Bernard & Pease 1969, Bonucci 1971, Irving 1973) Current data indicate that acidic phospholipids, associated with the mechanism of calcification, play an important role in binding of the calcium salts within the groundsubstance. If the matrix vesicles are the loci for initial calcification, the acidic phospholipids, within the vesicle membrane, provide sites for initial calcium binding and establish an environment in which mineralization can be facilitated (Irving 1976; Vogel & Boyan-Salyers 1976).

The inorganic material which accounts for about 65% of the bone substance, largely consists of minerals In the initial phase, calcium phosphate compounds are of an amorphous nature and are referred to as clusters. These calcium phosphate concentrations serve as nucleation centres It is still obscure which ion involved in the formation of these clusters is first present at the site of the nucleation According to Glimcher & Krane (1968) the phosphate ion is the first to reach the site. Others maintain that it is the calcium ion (Heeley & Irving 1973, Urist et al. 1964; Urry 1971) In vitro experiments had already shown that, in a solution which is metastable for calcium and phosphate ions, spontaneous nucleation (if any) was exceedingly slow; addition of collagen substantially accelerated the initial phase of nucleation. It should therefore be assumed that, at least in vitro, collagen can act as a catalyser (Bachra 1967, Bachra 1970)

Several observers have reported that these clusters, dependent on environmental influences, can persist and then mature to form crystals In the crystalline form, the calcium phosphate compounds present themselves as apatite crystals of the formula, $Ca_{10}(PO_4)_6(OH)_2$ (Bachra 1967, Eanes et al 1965; Termine & Posner 1966; Termine et al. 1967, Wadkins et al. 1974) The biological fluids in vivo are likewise metastable for calcium and phosphate ions During in-vivo mineralization it was established by Wergedal & Baylink (1974) that a rapid initial phase, with cluster formation, preceded a slow phase in which crystalline structures were formed

Present knowledge of the various bone constituents has failed to supply an answer to the question why bone matrix, unlike other tissue matrices, is mineralized in vivo. Fleisch and co-workers have formulated a possible solution to this problem. In human serum and urine they discovered a mineralization inhibitor which, upon analysis, proved to be pyrophosphate (Fleisch & Neuman 1961; Fleisch & Russell 1970) It was established in in-vitro experiments that pyrophosphate, in low concentrations, can block the formation and maturation of crystals (Fleisch 1964). The occurrence or non-occurrence of mineralization, therefore, is partly determined by the local pyrophosphate concentration This concentration can be reduced by the alkaline phosphatase, which has a pyrophosphatase activity (Cox &

Griffin 1965; Eaton & Moss 1966; Eaton & Moss 1968; Wöltgens et al. 1971).

Summary

The processes involved in the mineralization are not yet fully understood, but several investigators are convinced that the formation of nucleation centres is of fundamental importance in this process, and that the bone cell is an indispensable contributor. These nucleation centres can develop in collagen but also outside collagen. To collagen a catalyzing role in the formation of nucleation centres has been assigned. Furthermore it is strongly suggested that extracellular matrix vesicles could be loci for initial mineral deposition. There appeared to be convincing indications that pyrophosphate and pyrophosphatase are important for the regulation of the mineralization process. The diagram in fig. 3 is an attempt to schematize the complex events of the mineralization process.

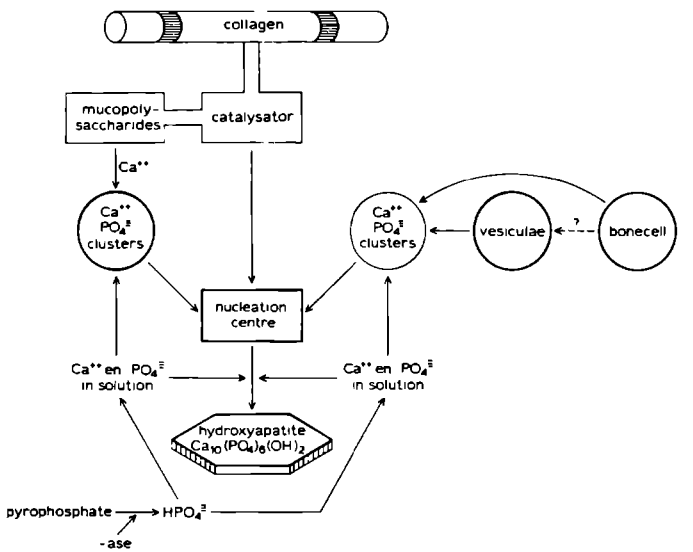


Fig. 3. Diagram of the possible course of mineralization (for explanation see text).

INDUCTION OF HETEROTOPIC OSSIFICATIONS

Introduction

The term induction is used with reference to differentiation which occurs in a tissue as a result of a physicochemical effect produced by another tissue or by a variety of agents. The concept was introduced in 1912 by the embryologist Spemann (review: Spemann 1938). Numerous embryological induction systems have since been described. An induction system for embryological skeletal tissue, for example, was elucidated by Schowing (1968).

If in an induction system competent cells differentiate into osteoblasts, chondroblasts and haemocytoblasts, then positive bone induction is present (Holtfreter 1933). This process can take place at a heterotopic site, in which case it is defined as positive heterotopic bone induction. In view of the experiments to be carried out, this chapter discusses heterotopic bone induction in particular.

An inductor is required to start an induction process, and several authors have described the existence of extracellularly localized inductors (Ave et al. 1968; Grobstein 1964). According to Waddington (1966), however, there are also intracellular inductors which can cause auto-induction within a cell.

Several induction systems for experimental heterotopic ossifications are discussed in the literature. The use of vital and devitalized tissue as inductor is a prominent feature of these publications.

Vital inductor

It has been known for decades that the epithelium of the urinary and biliary tract is capable of starting bone induction. Several autotransplantation experiments in dogs have demonstrated this (Bell & Knudtson 1961; Huggins 1931; Huggins & Sammet 1933; Sacerdotti & Frattin 1902). They also showed that induction does not occur in all tissues. After autotransplantation of urinary tract epithelium in dogs, positive bone induction was observed in muscle and fascia, but not in kidney, liver and spleen (Huggins et al. 1936). Anderson et al. (1964) and Wlodarski et al. (1970) demonstrated a bone-inducing capacity in certain human amnion cell lines. In their experiments it also became clear that transplantation of vital inductors is subject to the laws

of the immunological system. The degree of resulting bone induction partly depends on the immunogenicity of the inductor. After heterotransplantation, for example, only very moderate bone induction occurs. Suppression of the immune reaction by means of cortisone causes substantial improvement. (Wlodarski et al. 1971a; Wlodarski et al. 1971b).

Vital autologous skeletal transplants have also been successfully used as inductor. Urist & McLean (1952) introduced vital skeletal transplants into the anterior chamber of the eye in rats, and observed unmistakable ossification. Studies by Ray & Sabet (1963) and Thorogood & Craig Gray (1975) showed that the ossification results both from proliferation of transplanted bone cells and from tissue cells of the host induced by the transplant. After autologous heterotopic transplantation of vital bone marrow it is more difficult to interpret the origin of the newly formed bone. According to various authors, several types of bone-forming cells are transplanted in that case: genetically determined osteoblasts, and as yet undifferentiated cells which, in response to the inductor in the transplant can begin to function as osteoblast. Another possibility is that induction is effectuated by the transplant on the host tissue cells, although this has not so far been demonstrated with certainty (Amsel & Dell 1972; Burwell 1966; Friedenstein et al. 1966; Friedenstein et al. 1968; Simmons et al. 1973).

In order to be certain that an induction process is responsible for heterotopic ossification, the use of devitalized skeletal transplants is imperative (Bridges & Pritchard 1958; Ostrowski & Wlodarski 1972) for, because no vital cells are transplanted, any resulting positive heterotopic ossification can be attributed to the inducing capacity of the transplant.

Devitalized inductor

The use of devitalized tissues as bone inductors is limited to skeletal transplants. It is generally assumed that devitalized epithelium of the urinary and biliary tract, or homogenates or extracts of this epithelium, have no bone-inducing capacity (Ostrowski & Wlodarski 1972).

It was demonstrated very early by Urist & McLean (1952) that freezing enables devitalized osseous autotransplants to induce heterotopic ossification. Subsequent detailed studies focused on the results obtained with devitalized homologous and heterologous transplants. With avital homologous transplants, several investigators observed positive heterotopic bone induction (Bridges 1959; Campbell et al. 1953; Chalmers et al. 1975; Urist & McLean 1952). As in the case of vital inductors, the bone inducing properties depend partly on the antigenicity of the transplant. A slight degree of antigenicity can favour bone induction (Bang 1972; Simmons et al. 1974; Urist et al. 1967). An argument in favour of this hypothesis was the observa-

tion made by Koskinen et al. (1972) and Thompson & Urist (1970) that administration of cortisone reduced bone induction in response to a homologous transplant The poor results obtained with heterologous transplants may be due to excessive transplantation reactions: the transplant is encapsulated by sterile inflammatory tissue, which precludes contact with the host tissue cells to be induced (Simmons et al. 1974). Several investigators have pointed out that excessive antigenicity unfavourably affects the degree of bone induction. The antigenicity of transplants can be kept within reasonable limits by lyophilization (Chalmers 1959, Heiple et al. 1963; Ostrowski 1969, Urist & Craven 1970).

Transplants can be devitalized in various ways, but it should be borne in mind that the processing of the transplants is one of the factors which determine its inducing capacity (Heiple et al. 1963). Efforts were made to find a method of devitalization which would leave the inducing capacity of the transplant intact In 1965 Van der Putte & Urist (1965) described a method of devitalization which made use of decalcifying agents. Homologous transplants decalcified with diluted hydrochloric acid were implanted intramuscularly in various animal species. A reproducible high rate of positive bone induction was scored. Several experiments revealed that the inducing properties of the inductor are highly dependent on the temperature, the duration of the decalcification process, and on the appropriate concentration of hydrochloric acid (Van der Putte & Urist 1965; Urist et al. 1967; Urist et al. 1968)

There remains the intriguing question: which constituent in the decalcified transplant acts as inductor? In numerous experiments Urist and his coworkers have attempted to analyse the inducing substrate in the decalcified matrix. This matrix is, in competent tissue, capable of inducing bone which becomes organized as lamellar bone with, in the medullary space, haemopoietic bone marrow In 1970 Urist advanced a working hypothesis in which the inductor was defined as Bone Morphogenetic Protein (BMP) (Urist 1970a; Urist 1970b, Urist & Strates 1971)

Bone Morphogenetic Protein (BMP)

The tentative conclusion from the various experiments was, the bone-inducing capacity is the function of a separate polypeptide closely bound to, but not an essential constituent of collagen. Tyrosine and phenylalanine are probably important components of BMP (Bang & Johannessen 1972; Iwata & Urist 1972; Urist & Iwata 1973; Urist et al. 1973; Urist et al. 1974a). The inducing capacity was found to diminish with decalcification at a neutral pH, unless one reduced the temperature and/or added enzyme inhibitors There are indications that this observation should be ascribed to the activity of an endogenous enzyme, probably a neutral peptidase, which was referred to as

Bone Morphogenetic Proteinase (BMP-ase) (Urist et al. 1972; Urist & Iwata 1973; Urist et al. 1974a; Urist et al. 1974b).
There is a strongly motivated suspicion that readily soluble proteins not localized in collagen, can exert a regulating influence on the BMP/BMP-ase balance (Leaver et al. 1975; Mikulski & Urist 1974; Urist 1973). This hypothetically formulated process, however, requires extensive further evidence It has so far been impossible to extract and chemically analyse the substances described.
A comprehensive survey of alternative hypotheses on bone induction has been given by Ostrowski & Wlodarski (1972). Several investigators have focused attention on a possibly important role of electric currents in the induction of bone tissue (Bassett 1968; Bassett 1972; Becker & Murray 1967; Pritchard 1972). De Groot (1973) advanced a hypothesis which brought the activities of BMP and electric currents under a single denominator. His starting-point was that BMP has a positive charge. In an electric field, storage of BMP should occur on the cathode side and this might explain differentiation of mesenchymal cells to osteoblasts on the cathode side.
The manner in which BMP exerts its influence on the cell to be induced is still obscure. Urist and his co-workers postulate a physicochemical contact between BMP and receptor sites in the microvilli of the cell membrane. This might start an unknown intracellular process by which the cell is given a gene expression capable of osteogenesis and chondrogenesis (Urist & Strates 1971; Urist et al. 1974a). Direct contact between the matrix which contains BMP and the cell to be induced seems to be required. Experiments with diffusion chambers in vivo have shown that there is no diffusion of BMP from the matrix (Büring & Urist 1967a; Urist et al. 1967).
Induction takes place in as yet undifferentiated mesenchymal cells. Cells which as a result of the mechanism of induction start to function as bone-forming cells, are referred to as osteogenetically competent.

Osteogenetic competence
Mesenchymal cells are called osteogenetically competent if the gene systems which regulates the formation of a calcifiable matrix are present. Osteogenetic competence can therefore be defined as a not yet activated ability of the cells to differentiate into bone cells. The induction mechanism is believed to be responsible for the activation of these gene systems (Firschein & Urist 1972; Urist & Strates 1971). The experiments of Urist et al. (1969) revealed that mesenchymal cells in different tissues differ widely in osteogenetic competence. After implantation of decalcified homologous transplants, marked induction was observed in muscle, tendon, subcutaneous tissue, pericardium and lungs. Only moderate induction was observed in testes, pancreas and

ovaries, and no induction occurred in lymph nodes, adrenal glands, thyroid, liver, spleen and kidneys. Yet it is possible to effect heterotopic bone induction in, say, the kidney For this purpose osteogenetically competent tissue such as muscle should be implanted along with the transplant, or the transplant should first be implanted in muscle tissue and, after a certain period, removed and re-implanted in the kidney (Chalmers et al. 1975, Urist et al. 1969).

A possible explanation of the difference in osteogenetic competence could lie in the presence or absence of osteogenesis-inhibiting factors in the various tissues. This hypothesis is based on various experiments (Bridges & Pritchard 1958; Chalmers 1959; Chalmers et al. 1975; Urist et al. 1969). For example, during foetal extraskeletal erythropoiesis, haemopoietic marrow without bone is present in spleen, liver and lymph nodes (Owen 1970). This might imply the possibility of protection from ossification in the abovementioned organs, even though this cannot be absolute, for an autologous bone transplant implanted in the spleen, continues to grow (Chalmers & Ray 1962). The findings reported by Friedenstein (1968) and Rosin et al. (1963) also argue in favour of the existence of such factors. In experiments in diffusion chambers it was demonstrated that isolated spleen cells are osteogenetically competent, whereas splenic tissue placed in the diffusion chamber is not. These factors can be simply brought under the heading of milieu factors. The tissue milieu plays an important regulating role at the start and in the course of the induction process. The importance of adequate humoral, mineral, metabolite and oxygen supplies has been demonstrated (Urist et al. 1967). In vitro experiments have confirmed the influence of the milieu on the induction process (Nogami & Urist 1970; Nogami & Terashima 1976; Terashima & Urist 1975; Urist et al. 1973)

Yet another aspect of the induction process requires further elucidation. In their microscopic study of the induction process, Urist and his co-workers observed the following regularity. During the first few days the transplant is surrounded by irregularly shaped cells such as histiocytes, macrophages and polynuclear giant cells. These cells develop and present themselves light-microscopically as osteoclasts According to Urist, these cells are of essential importance in resorption of the matrix. No differentiation of mesenchymal cell to osteoblast occurs until resorption of the matrix has started. On the contrary it is possible for a mesenchymal cell to differentiate into a chondroblast without histological detectable resorption (Urist et al. 1967; Urist et al. 1968; Urist et al. 1969).

It was initially thought that the osteoclast and osteoblast involved in induction had a common precursor cell (Owen 1970; Young 1962; Young 1963). Reports by Buring (1975), however, necessitated a reconsideration of this

theory. In his opinion the osteoclast is a monocyte originating from the blood stream, which enters the tissue via diapedesis and then develops into an osteoclast. The cell which responds to the inductor by turning into osteoblast or chondroblast, however, originates from the perivascular mesenchyma. Histochemistry of frozen sections at various time intervals, after implantation, revealed a distinct pattern for the appearance of various enzymes. Quantitative analysis on tissue homogenates for alkaline phosphatase, acid phosphatase and lactic dehydrogenase corroborates the histological observations in relation to the present cells. This further indicates that no osteoblast activity occurs until resorption has taken place (Büring 1974).
The above considerations prompted the question whether the clinical use of decalcified homologous transplants is a possibility.

Clinical application
The use of decalcified bone transplants is by no means new. In 1889, Senn described a technique of using decalcified transplants to repair defects resulting from resection. His results, however, had been variable (Senn 1889). The discussion of transplants of this type ceased until the early Sixties, when Sharrard & Collins (1961) reported good results obtained with homologous bone transplants decalcified with the aid of EDTA (ethylenediamine-tetraacetic acid) in the treatment of children with scoliosis by spinal fusion.
For correct interpretation of the usually variable results, certain aspects merit a more detailed discussion. The decalcification procedure used probably led to total or partial loss of the bone-inducing capacity of the matrix, for decalcification is a very subtle procedure (Urist et al. 1968). In addition, a comparative study by Urist & Craven (1970) revealed an unmistakable species-specific difference in BMP-stability and sensitivity to bone induction. Both factors are determinants of the degree of bone formation.
The clinical use of homologous transplants decalcified according to Urist had so far been limited. Yet these transplants have been successfully used to repair dental defects resulting from periodontosis (Libin et al. 1975; Urist 1965). After orthotopic implantation, however, it is difficult to distinguish between bone formation caused by induction, and proliferation of existing tissue. At this time, the clinical use of decalcified homologous bone transplants is not a rational alternative to the use of autotransplants. This can only be considered once it has been proven that these homologous transplants can induce heterotopic bone formation in human individuals (Büring 1974).
Within the context of a possible clinical use it is necessary to focus attention on the current processing of bone bank transplants, which is not in the complete interest of the inducing capacity. These are sterilized by irradiation, and the dose applied amply exceeds the critical limit of 0.2 - 0.5 M rad, at

which inducing capacity is lost (Buring & Urist 1967b; Buring 1970) Ethylene oxide gas is an alternative without any untoward effect on BMP (Buring 1969) The possible inducing capacity of homologous bone transplants can only come into its own after the appropriate decalcification procedure This, however, would affect the supporting function of the transplant, it was believed. A study by Dubuc & Urist (1967) has meanwhile revealed that superficially decalcified transplants can likewise be biologically active as inductors.

Summary

It can be concluded that there are three prerequisits for heterotopic bone induction: 1) an inducing stimulus; 2) an osteogenetic precursor cell; 3) a tissue milieu susceptible to osteogenesis.

We discussed the experimental use of decalcified homologous bone transplants as inductors. The decalcification procedure tested by Urist and his co-workers proved not or hardly to affect the inducing capacity of the transplant - a capacity which has been hypothetically attributed to a Bone Morphogenetic Protein (BMP), of which the hitherto discovered characteristics were discussed. It is assumed that, after implantation, contact with BMP precedes the induction of a suitable osteogenetic precursor cell.

A precursor cell which is suitable for induction, is the mesenchymal cell that is found in all tissues. It has been demonstrated that mesenchymal cells in different tissues differ markedly in sensitivity to bone induction. This difference is referred to as a difference in osteogenetic competence It is conceivable that this difference in sensitivity to bone induction, between different tissues, depends on the presense or absence of a milieu susceptible to osteogenesis. The nature of the relevant factors in the milieu has so far remained a matter of speculation.

DIPHOSPHONATES; PHYSIOLOGICAL AND CLINICAL ASPECTS

In the early Sixties, increased knowledge of the physiological events involved in calcification of bone matrix prompted the question why only bone matrix calcifies in vivo, while other matrices containing collagen, e.g. the skin, do not or occasionally under pathological conditions in vivo or artificial conditions in vitro.

From technical chemistry it has been known for some decades that condensed phosphates, consisting of P-O-P complexes, are able to prevent precipitation of calcium carbonate in a solution. This ability makes them useful as water softeners (Buehrer & Reitemeier 1940).

A comparable substance, pyrophosphate (PP_i), was found in plasma and urine and was identified as a calcification inhibitor (Fleisch & Neuman 1961). Pyrophosphate is an inorganic phosphate which consists of P-O-P complexes (fig. 5). It is present in biological fluids along with other calcification-inhibiting factors, and probably has a special affinity for bone tissue (Feagin et al. 1969; Jethi et al. 1970). The action of pyrophosphate was further tested in in-vitro experiments.

Pyrophosphate action in vitro

It is generally accepted that PP_i can invade the hydration layer which surrounds the hydroxyapatite crystals, and is bound by the crystals at suitable sites. There are indications that calcium invades the layer of water along with PP_i, and that phosphate is released from it (Jung et al. 1973; Robertson & Morgan 1971). Several publications (e.g. by Fleisch and co-workers) describe that low concentrations of PP_i are able to inhibit precipitation of calcium phosphate in a solution in vitro. It also blocks the conversion of amorphous calcium phosphate into crystalline hydroxyapatite (Francis 1969). In addition they are able to inhibit the dissolution of existing calcium phosphate compounds (Fleisch & Neuman 1961; Fleisch et al. 1966a; Fleisch et al. 1966b; Fleisch et al. 1968a; Francis 1969). In tissue cultures, too, PP_i in serum concentration was demonstrated to be able to inhibit mineralization (Fleisch et al. 1966c). A contrasting observation was that resorption caused by parathormone in tissue cultures cannot be inhibited by PP_i (Russell et al. 1970b).

Although PP_i is known to be subject to rapid hydrolysis, this difference cannot be readily explained Jung et al (1974) suggested that PP_i can still reach sites of mineralization but not sites of resorption as a result of hydrolysis, however, they failed to present convincing arguments in support of this hypothesis

The question remained whether an equivalent function of PP_i could be demonstrated in vivo.

Pyrophosphate action in vivo

In view of the effects of PP_i on calcium phosphate compounds in vitro and the verified presence of PP_i in biological fluids, it is quite possible that PP_i protects soft tissues from calcification In addition PP_i, because it is subject to hydrolysis, could be one of the determinants of the start of physiological calcification (Fleisch & Russell 1970; Orimo et al 1971, Robertson & Morgan 1971). In order to investigate these possibilities, in-vivo experiments were made which were not very successful, except for the observation made by Schibler et al. (1968) that PP_i, if given parenterally, can prevent induced ectopic calcifications The poor results should be ascribed to the rapid hydrolysis of PP_i given in vivo. Several authors have pointed out that inorganic pyrophosphate hydrolyses very easily as a consequence of changes in temperature and pH as well as enzymatic activity (Francis 1969; Robertson & Morgan 1971). Further analysis showed that the involved enzyme system was alkaline phosphatase which, at physiological pH, has a pyrophosphatase activity (Cox & Griffin 1965; Eaton & Moss 1968; Felix & Fleisch 1974, Woltgens et al. 1971) It is exceedingly difficult to prove the correctness of the hypothesis that PP_i is directly involved in calcification in vivo It has so far been impossible to determine PP_i concentrations in skeletal tissues during the various stages of resorption and mineralization (Fleisch et al 1968a). The manner in which PP_i plays its possible role is likewise a matter of speculation. Fleisch and co-workers postulated a direct relation between PP_i and hydroxyapatite crystals. PP_i should protect the attainable sites of mineralization and further growth of the crystal and its resorption cannot take place until after hydrolysis of the PP_i (Fleisch et al 1968a; Fleisch & Russell 1970) (Fig. 4).

In an in-vitro study with embryonic chick femurs Anderson and Reynolds (1973) showed that the matrix vesicles are the initial site of crystal deposition. Addition of inorganic pyrophosphate to this medium caused a marked increase in calcium uptake in the vesicles, which are known to be rich in pyrophosphatase It is suggested that vesicles induce calcification by concentrating calcium and phosphate by the local destruction of pyrophosphate (Felix & Fleisch 1976)

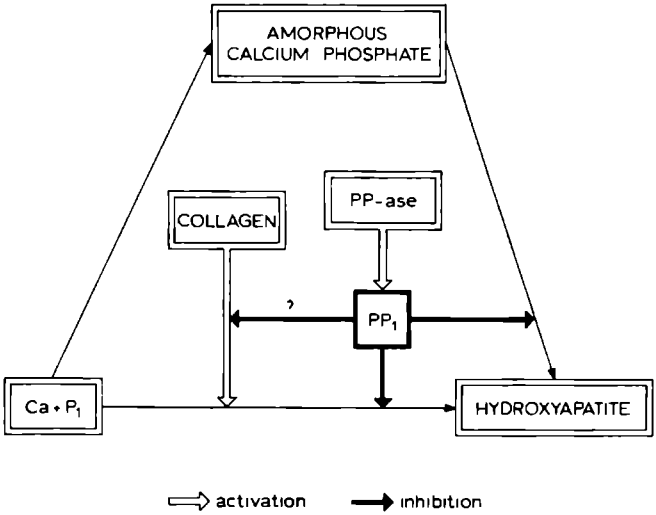


Fig. 4. Possible role of pyrophosphate in calcification. PP-ase indicates pyrophosphatase; P_i inorganic phosphate; PP_i indicates inorganic pyrophosphate; and Ca calcium.

In addition, there are clinical facts which corroborate a possible role of PP_i in calcification. For example, an excessive plasma PP_i concentration was found in disorders of mineralization associated with hypophosphatasia or renal insufficiency (Fleisch et al. 1971; Russell et al. 1969; Russell et al. 1971). Pseudogout (chondrocalcinosis), in which calcium pyrophosphate crystals develop within the joint, could be an expression of a local disorder of PP_i metabolism (Jacobelli et al. 1973; Russell et al. 1970a). For further elucidation, mention must be made of the findings of Russell & Fleisch (1969) and Hausmann et al. (1970). They came to the conclusion that prevention of renal calculi and caries is partly dependent on a sufficient concentration of PP_i in urine and saliva, respectively.

It is evident, however, that clinical therapeutic use of PP_i is impossible, because of the rapid hydrolysis of PP_i after oral as well as after parenteral administration (Jung et al 1970) Other polyphosphates, with a P-N-P bond instead of a P-O-P bond, proved to have the same disadvantage in experiments (Robertson & Fleisch 1970) Some diphosphonates, with a P-C-P bond (fig. 5) on the other hand have an action which seems to resemble that of PP_i; moreover, they are resistant to hydrolysis The P-C-P complexes are very stable, and so far no enzyme able to catalyse their degradation has been found in higher organisms (Russell et al 1973) In some micro-organisms, however, phosphonatases have been identified which can cause disintegration of certain P-C-P complexes (La Nauze et al 1970).

The two diphosphonates so far intensively studied and tested are:

- EHDP : ethane-1-hydroxy-1, 1-diphosphonic acid.
- Cl_2MDP : dichloromethylene-diphosphonic acid.

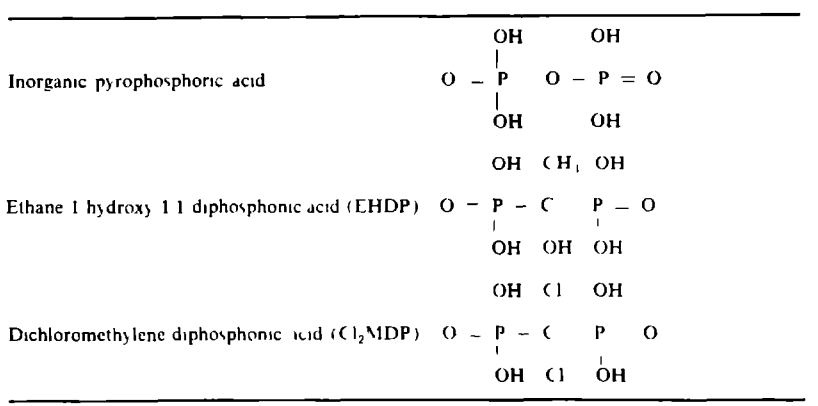


Fig 5 The formulae (as acids) of pyrophosphate and two diphosphonates

Studies with EHDP have shown that absorption chiefly occurs in the stomach and no degradation takes place in the digestive tract. The degree of absorption in the stomach varies with the species and ranges from 1% to 10% for rats, rabbits and human individuals. In this context it has to be noted that this percentage may be higher in growing animals, and that interindividual variations are possible (Michael et al 1972). There are no indications that EHDP is metabolized. About one-half of the EHDP absorbed, is taken up by bone, a very small amount by the soft tissues, and the remainder is excreted unchanged in the urine (Michael et al 1972) Besides the glomerular filtration there may be a tubular secretion of EHDP (Trohler et al. 1973). King et al

(1971) and Michaël et al. (1972) determined the half-life of EHDP in dogs and rats with the aid of C^{14}-labeled EHDP. It was found to be about 12 days after oral and 30 days after subcutaneous administration. In clinical application, if any, the problem will therefore be to plan a good dosage scheme to offset the disadvantage of the poor and variable absorption after oral administration. Tests have so far revealed little evidence of possible toxic side effects. Long-term administration to rats and rabbits caused no disturbance of reproductive function, and no embryotoxic effects have so far been demonstrable (Nolen & Buehler 1971). In human subjects, however, several investigators found hyperphosphataemia at an oral dosage of about 20 mg/kg/day (Bijvoet et al. 1974; Gunčaga et al. 1974; Recker et al. 1973; Smith et al. 1971). It was suggested that an increased tubular resorption occurs as a result of a direct influence of EHDP on the tubular cells and causes the hyperphosphataemia. The renal tubules continued to respond normally to parathormone (Bijvoet et al. 1974; Recker et al. 1973).

Diphosphonate action in vitro

It has been demonstrated in vitro that diphosphonates have the same action as PP_i: both block the conversion of amorphous calcium phosphate to hydroxyapatite crystals and inhibit the growth and the dissolution of existing crystals (Fleisch et al. 1968b; Fleisch et al. 1969; Francis 1969; Russell et al. 1970b). These actions are due to a strong binding of the diphosphonates to the surface of the crystals (Jung et al. 1973; Meyer & Nancollas 1973). As in the case of PP_i calcium is taken up in, and phosphate released from the hydration shell (Robertson et al. 1972). Numerous studies have shown that diphosphonates, in contrast to PP_i, can inhibit bone resorption in several experimental systems. In tissue culture, they can prevent the resorption induced by parathyroid hormone (PTH) in mouse calvaria whether measured morphologically (Russell et al. 1970b) or by release of ^{45}Ca from prelabeled bone (Reynolds et al. 1972). At equal doses Cl_2MDP is more potent than EHDP. Although Cl_2MDP and calcitonin are similar in their effect on release of ^{45}Ca, there are differences in other respects. Thus Cl_2MDP blocks the PTH-induced increases in acid phosphatase and acid pyrophosphatase, but calcitonin does not (Morgan et al. 1973).

Diphosphonate action in vivo

According to Fleisch et al. (1970) it can be stated in general that a reliable relation exists between the potency of diphosphonates to inhibit crystal growth in vitro and their potency to prevent soft tissue calcifications in vivo. EHDP, given at high doses to several species, leads to appearance of unmineralized osteoid tissue in preexisting bone (Jowsey et al. 1970; King et al. 1971;

Russell et al. 1973; Schenk et al. 1973). In growing animals this is associated with inhibition of cartilage mineralization and the persistence of hypertrophic cartilage cells. The ephiphyseal plate becomes wider and radiologically resembles classical vitamin D deficiency rickets (Schenk et al. 1973). Histologically, however, the two conditions differ since the vitamin D deficient cartilage shows an accumulation of proliferating cells, while the cartilage from chicks treated with EHDP consists mainly of hypertrophic cells (Bisaz et al. 1973).

One interesting observation was that intestinal calcium absorption diminishes in response to EHDP. It was found that the diminished absorption was due to decreased synthesis of dihydroxycalciferol (1,25 DHCC) in the kidney (Hill et al. 1973); dihydroxycalciferol is the active vitamin D_3 metabolite involved in intestinal calcium absorption (Bonjour et al. 1973a; Bonjour et al. 1973b). An argument in favour of an indirect effect of EHDP on the intestinal mucosal cell is the finding reported by Bonjour et al. (1972) that the concentration of calcium-binding protein and that of the enzyme Ca-ATP-ase (involved in absorption) in the mucosal cell decreases. Both concentrations, after all, are controlled by vitamin D (Melancon & De Luca 1970; Wasserman & Taylor 1966).

The dose of EHDP required to impair bone mineralization varies according to the species, the duration of treatment and the route of administration. Roughly, it is seen at doses above 4 mg EHDP/kg/day given parenterally for short periods (King et al. 1971). However, parenteral administration to rats of less than 10 mg/kg/day for 14 days, and to dogs to less than 8 mg/kg/day for 28 days does not change the bone mineral concentration. The effect of EHDP on bone mineralization is more impressive in growing than in adult animals, and more accentuated in trabecular bone than in the case of cortical bone (Rosenblum 1974a). All these effects of EHDP on the mineralization are reversible when administration is discontinued although only after a delay (Bijvoet et al. 1974; Gunčaga et al. 1974; King et al. 1971; Russell et al. 1973). Some observers demonstrated that the quantity of unmineralized osteoid tissue diminishes after a long-term administration of EHDP. Cabanela & Jowsey (1974) demonstrated a diminution of the osteoid-increase after 12 weeks EHDP-administration in adult dogs (5 mg/kg/day, s.c.). This statement was confirmed by Bijvoet et al. (1974). Cabanela & Jowsey (1974) suggest that this might be due to a feedback mechanism from the matrix on the osteoblast.

Very little is known about the quality of the osteoid tissue formed. Larsson (1974) observed an intracellular change in precollagen synthesis in odontoblasts in response to EHDP. This synthesis is partly regulated by an enzyme, which requires also calcium as a co-factor. Larsson suggested that EHDP might indirectly interfere with collagen synthesis by trapping calcium. He

was also able to demonstrate an increase of the proteoglycans in the calcifying zone in rats treated with high doses of EHDP A degradation of the macromolecular proteoglycan complexes might be an integral part of the calcification process. This observation suggests that EHDP might interfere with calcification also at this level (Larsson & Larsson 1976)

EHDP administered in vivo has also an effect on the bone resorption A small dose (about 1-4 mg EHDP/kg/day, subcutaneously) caused inhibition of bone resorption in rats (King et al. 1971; Rosenblum 1974b, Russell et al. 1973). A finding, which corroborates this information, is that small doses of EHDP can inhibit osteoporosis which is experimentally induced in rats by means of immobilization (Jowsey & Holley 1973, Lane & Steinberg 1973; Michael et al. 1971).

In-vivo findings, reported for Cl_2MDP, differed from those for EHDP in that only resorption-inhibiting competence was established (King et al 1971; Minkin et al 1974, Russell et al 1973); in this respect, however, Cl_2MDP was found to be superior to EHDP (Gasser et al. 1972; Reynolds et al. 1972) Even with larger doses of Cl_2MDP, no inhibition of mineralization was obtained; since resorption was markedly inhibited, however, densification of bone tissue occurred (Michael et al 1971; Reynolds et al. 1973) Consequently the proliferation of unmineralized matrix which follows EHDP administration, was not observed. Harris & Heaney (1969) suggested the possibility of a depression in matrix synthesis secondary to inhibition of resorption. This depression might be prompted by a signal which relates the degree of matrix formation to the degree of resorption The simplest explanation for the effect of EHDP on the mineralization process is, that it prevents the formation of apatite crystals at mineralization sites by a direct action on crystals. The critical dose at which EHDP will interfere with deposition of crystalline hydroxyapatite has been calculated to correspond to a dose of 5 mg EHDP/kg body weight, parenterally (King et al. 1971).

However, other modes of action are possible. There is good reason to believe that the matrix-vesicles play a role in the initiation of the calcification process (Anderson 1973). Inhibition of apatite formation in these vesicles was a prominent feature in EHDP-treated rats (Larsson & Larsson 1976). The same observation was made by Anderson & Sadjera (1976). This inhibition would be expected to result in a lack of mineralization of the surrounding matrix. It has been suggested that low concentrations of PP_i promote the earlier stages of calcium accumulation by the vesicles (Anderson & Reynolds 1973). EHDP might compete for PP_i at this site, and thereby block calcification (Rasmussen & Bordier 1974c).

Guilland et al. (1974) carried out an in vitro-experiment with rat kidney cells. These authors described an accumulation of calcium in the mitochondria in

response to EHDP, and found evidence in support of the assumption that EHDP may interfere with membrane transport of calcium.
An additional possibility is that EHDP blocks the enzymatic degradation of PP_i (Woltgens et al. 1973, Woltgens 1974). He demonstrated the competitive inhibition of a PP_i-ase from hamster molars by EHDP, which can form a complex with magnesium ions, a co-factor of the enzyme.
At present, however, it seems hardly possible to single out any specific factor by which the changes observed in EHDP-treated animals might be explained.
The literature comprises incidental reports describing changes in the aspect of the bone cells in response to EHDP. Doty et al (1972) described an electron-microscopically established decrease in the lysosome concentration of osteocytes in rat bone treated with EHDP. Schenk et al (1973) reported unmistakable light-microscopic changes in the aspect of the osteoclasts in rat bone treated with EHDP: the cells became larger, the number of nuclei increased and the cytoplasm showed less chromaffinity. Examination with the electron microscope revealed a decrease in the percentage of active osteoclasts in response to EHDP (Schenk 1974). Rowe & Hausmann (1976) demonstrated a significant increase in the incidence of these osteoclast abnormalities accompanied with a significant inhibition of ^{45}Ca release. They suggest a specific morphological effect of EHDP on osteoclasts, because there was no change in the morphology of the surrounding non-osteoclast cells. Some authors, however, described the persistence of hypertrophic epiphyseal chondrocytes under the influence of EHDP in vitro (Bisaz et al. 1973) and in vivo (Larsson & Larsson 1976).
Although there are incidental descriptions of sometimes hardly specific changes at the cellular level, these findings do not prove a direct influence of diphosphonate on bone cell metabolism. It is to be borne in mind, moreover, that changes in the solubility of bone minerals, and therefore in local ion concentrations, can likewise lead to secondary changes (Rasmussen et al. 1970).

Clinical application

In view of all these experimental findings, it seems likely that diphosphonates can have potential clinical value. At this time EHDP is the diphosphonate most widely studied and tested, and the only one so far used in human individuals. This potential value was concluded from the results obtained in animal experiments in vivo. Induced soft tissue calcifications can be prevented by administration of EHDP (Chalmers et al 1975; Francis et al. 1972; Schibler et al. 1968; Strates et al 1971). In an experiment on rats, the preventive effect of EHDP on the formation of bladder calculi was demonstrated (Fraser et al. 1972).

One of the best-known clinical syndromes associated with ectopic ossifications is myositis ossificans progressiva, in which extensive ectopic ossification is observed. Patients with this disease are few, as are the therapeutic possibilities. Patients of this category who received doses of EHDP (20 mg/kg/day/by mouth) showed an improvement in more than half of the cases (Geho & Whiteside 1973). In many cases, however, the improvement is restricted to an increased mobility in the affected joints (Smith et al. 1976). In a few adult patients the periarticular ectopic bone was surgically removed under EHDP-protection, without subsequent relapse (Russell et al. 1972). EHDP was used in the treatment of a small number of patients with ectopic calcifications associated with scleroderma, dermatomyositis and calcinosis universalis. The results in these cases were not promising. The literature comprises a report on one patient wich calcinosis universalis who responded well to EHDP-medication (Cram et al. 1971).
An unmistakable preventive effect of EHDP was demonstrated in periodontosis, in which EHDP can prevent plaque and calculus formation (Mühlemann et al. 1970; Sturzenberger et al. 1971). Recurrence of calculi in the urogenital tract was prevented by a dose of EHDP of 20 mg/kg/day, by mouth (Baumann et al. 1974).
In this section mention should also be made of the study by Bijvoet et al. (1974), from which an important motivation for our animal experiments resulted. In a comparative study they found that EDHP (20 mg/kg/day, by mouth) could prevent the periarticular ossifications sometimes observed after total hip replacement. The patients were given EHDP from 6 weeks before to 6 - 12 weeks after the operation. It was also found that ossifications could develop after discontinuation of EHDP. In any case the clinical benefit was evidently improved, less painful mobility.
Beside the use of EHDP in ectopic ossifications, it has been applied to patients, suffering from M.Paget, with promising results (Altman et al. 1973; Russell et al. 1974; Russell & Fleisch 1975; De Vries 1975).

Summary
This chapter discusses the physiological action on the mineralization process of pyrophosphate (PP_i) and diphosphonates, structural analogues of pyrophosphate. The in-vitro findings with PP_i on mineralization cannot be directly extrapolated to in-vivo experiments. A possible biological role of PP_i in mineralization is discussed in more detail. The action of PP_i is based on its special affinity for hydroxyapatite crystals, but an additional possibility is that it might interfere with the cellular and vesicular metabolism.
The action of diphosphonates is also discussed with reference to in-vitro experiments. An unmistakable effect of diphosphonates on mineralization is

demonstrable in vivo Whether or not the diphosphonates combine their affinity for hydroxyapatite crystals with an influence on bone cell metabolism is still questionable. Some indications in this direction are discussed.

DESCRIPTION OF THE EXPERIMENT

Introduction

The etiology of periarticular ossification after total hip replacement has so far remained obscure (Slooff 1973). In principle, differentiation can occur in periarticular, osteogenetic competent mesenchymal cells, thus creating a cell system capable of producing complete bone (Nollen & Slooff 1973; Urist et al. 1969). The differentiation might be induced by a physicochemical stimulus arising from the implant and/or from the procedure of implantation. Changes in the milieu, after all, can be among the determinants of differentiation (Urist et al. 1967).

On the other hand it is possible that vital bone cells enter the periarticular tissue during the operative procedure. These cells, however, might combine a proliferative potency with a potency to act as inductor in osteogenetic competent tissue (Ray & Sabet 1963; Thorogood & Craig Gray 1975).

The formation of periarticular bone after total hip replacement should therefore be defined as positive bone induction determined by several factors (Holtfreter 1933).

A clinical trial described by Bijvoet et al. (1974) revealed that administration of disodium-ethane-1-hydroxy-1,1-diphosphonate (EHDP) was able to prevent these periarticular ossifications. It was also found, however, that ossifications can still occur after discontinuation of EHDP.

Doses of EHDP, as given in this trial, cause inhibition of mineralization and lead to the appearance of unmineralized osteoid tissue in preexistent bone. Similar findings were reported by King et al. (1971), Rosenblum (1974b), Russell et al. (1973) and Schenk et al. (1973). Several authors, however, indicated that the osteoid proliferation is reversible after discontinuation of EHDP (Bijvoet et al. 1974; Cabanela & Jowsey 1974; Gunčaga et al. 1974; Russell et al. 1973).

The need for an evaluation of the influence of EHDP on the behaviour of an induced, osteogenetic competent cell system prompted the experiment to be described. A number of prerequisits determined our choice of the induction mechanism to be used in the experiment:

- a high degree of reproducibility;

- a devitalized inductor, which makes it possible after heterotopic implantation to study the influence of EHDP on bone formation, without a possible interference of existing bone in the vicinity;
- an induced osteogenetic competent cell system with and without the influence of EHDP should readily permit of comparison per unit of time.

The induction mechanism evolved by Urist and his co-workers seems to meet all these requirements.

Test animals

The rabbit was chosen as test animal for the induction model to be studied, because this animal is very sensitive to induction and application of the induction mechanism has proved to lead to positive bone formation in 90 - 100% of cases (Urist et al 1967; Urist et al. 1968; Urist & Craven 1970; Urist et al. 1970). The test animals used in the experiment were 125 young adult male rabbits of the New Zealand White strain. This strain was chosen because our animal laboratory had most experience with it At the start of the experiment the age of the animals ranged from 13.5 to 14.5 weeks and the average weight was 2.5 kg The rabbits came from litters with a limited number of sires. They arrived at the animal laboratory at least 14 days before the planned date of operation They were housed in individual pens with a grid floor, received water and libitum and were given a standard diet (standard mixture L.K. = Ol Hopefarms Ltd., Woerden, The Netherlands).

Inductor

Devitalized homografts were used as inductor. The donor rabbits (age 13.5 - 14.5 weeks) were sacrificed by cervical dislocation. From the diaphysis of the resected femurs, pieces with a length of 1 cm were cut and after the marrow was removed, immediately chilled to 0°. The entire procedure took 5 - 10 minutes.

The graft was devitalized by a carefully tested decalcification process, which minimizes te risk of a decrease in the inductive potency of the graft. For this purpose the fragments were decalcified in diluted hydrochloric acid (1 g bone per 100 ml 0.6 N HCl) under constant stirring during 48 hours at a temperature of 2°C, the hydrochloric acid being changed after 24 hours. After elution of the acid in 0.15 N NaCl, the fragments were deep-frozen to -70°C in a mixture of carbon dioxide and acetone. They were lyophilized and stored in an exsiccator with an exsiccant (Silicagel) at a temperature of 2-3°C until the time of implantation 2-3 days later. The procedure has been outlined by Chalmers (1959), Heiple (1963), Ostrowski (1969), Van der Putte & Urist (1965), Urist et al. (1967), Urist et al (1968), Urist et al. (1972) and Urist & Iwata (1973).

Site of implantation
The graft was implanted in the long lumbar back muscles. The mesenchymal cells in the muscular tissue have been shown osteogenetic very competent and highly sensitive to induction (Firschein & Urist 1972; Urist et al. 1969).

EHDP-dosage
The diphosphonate administered was disodium-ethane-1-hydroxy-1,1-diphosphonate (EHDP).* The effective dose was 15 mg $C_2H_8P_2O_7$ per 0.4 ml. The test animals were given 5 mg/kg body weight daily by intramuscular injection into the right lumbar region and gluteus maximus.

Operative technique
The test animals were fasted during 24 hours before the operation and anaesthetised by intravenous injection of 0.5 mg atropine and 0.5 ml fluanisone-phentanyl citrate (Hypnorm®, Philips Duphar B.V., Amsterdam). After intubation the posterior part of the left lumbar region was shaven and washed with Bethadine iodine soap. The animal was placed on the table on its right side, lying on a hot-water mattress, and connected to a Keuskamp Amsterdam Infantile Ventilator (AIV) for ventilation with a mixture of oxygen and nitrous oxide (ratio 1:2) and 0.5 - 1% halothane (Fluothane).
The operations were performed under sterile conditions. The left lumbar region was painted with iodine and then draped with sterile towels.
An incision of 2 - 3 cm was made in the posterior part of the lumbar region, about 2 cm proximal to the posterior crest and 2 cm lateral to the spinous processes. The distance was carefully maintained in order to avoid contact between graft and existing bone. In the same line the lumbar muscle fascia was incised, whereupon the lumbar muscle was divided with blunt scissors in the course of the muscle fibres. The graft was placed between the fibres, making sure that it was entirely surrounded by muscle tissue. The fascia was sutured with catgut and the skin with linen.
Before detubation the animal was given 1 mg nalorphine hydrochloride (Nourypharma B.V., Oss) by intravenous injection. After detubation the animal was placed near a radiator in order to prevent heat loss. The operation was exceedingly simple and readily reproducible.

Grouping
The test animals were divided into three main groups -A, B and C- for operation on different dates. In collaboration with the Mathematical Statistical Advice Department a list of random figures was used to ensure random distribution over six subgroups of 5 animals each, and some reserve animals (Kendall & Babington-Smith 1939).

* The EHDP was kindly supplied by Henkel & Cie, GmbH, Dusseldorf, West Germany.

The periods of observation in the three groups ranged from one to six weeks. It has been empirically established that rabbits can form complete bone in this way within six weeks (Urist et al. 1967; Urist et al 1968; Urist et al. 1969). Two smaller additional groups were formed – D and E –, in which the period of observation was substantially longer (8, 10 and 12 weeks).

Group A in this group the previously described induction mechanism was studied over a period of 6 weeks, for which purpose one subgroup per week was sacrificed (A_1 through A_6) This group actually served as control for the EHDP-treated animals (groups B and C).
Group B in this group the induction mechanism was studied over a period of 6 weeks under the influence of EHDP All animals in this group received 5 mg EHDP per kg body weight daily by intramuscular injection. Each week one subgroup was sacrificed (B_1 through B_6)
Group C. in this group the behaviour of the model after discontinuation of EHDP was studied after a period of 1 through 6 weeks (C_1 through C_6). All these animals were sacrificed on day 42 after implantation.
Group D: in this group the behaviour of the model over a longer period was studied, for which purpose one subgroup was sacrificed after 8, one after 10, and one after 12 weeks (D_8, D_{10} and D_{12})
Group E in this group we studied the question whether the changes caused by EHDP are reversible. All animals in this group were given 5 mg EHDP per kg body weight daily by intramuscular injection for the period of 4 weeks. One subgroup was sacrificed after 8, one after 10, and one after 12 weeks (E_8, E_{10}, E_{12}).

Disqualified animals
Animals disqualified in the course of the experiment were replaced by reserve animals if possible A total of 25 animals (20%) were finally excluded from the experiment, in which 125 animals were used. Eleven animals died an acute death. A postmortem dissection of 7 of these animals revealed the existence of a pneumonia. One animal was sacrificed because a broken back was diagnosed, and one because extensive haematomas had formed at the site of injection. Eleven animals had to be excluded from the experiment because it was found at sacrifice that an abscess had formed at the site of implantation (culture: Pasteurella multocida).
In the tables these animals are marked with an asterisk. One animal was excluded because the graft was not included in the sections cut.
The final arrangement of groups A, B and C is indicated in table 1, and that of groups D and E in table 2.

Table 1

group A 31 animals		group B 34 animals		period of observation	group C 30 animals		period of EHDP administration
0003		0062			0302		
0014		0077*			0310		
0020	A_1	0073	B_1	1 week	0319*	C_1	1 week
0028		0079*			0327		
		0087					
0004		0063			0303*		
0012		0065*			0305		
0031*	A_2	0071	B_2	2 weeks	0311	C_2	2 weeks
0029*		0096			0334		
0025		0090			0330		
0023		0088			0328		
0008		0084			0324		
0024	A_3	0082*	B_3	3 weeks	0322	C_3	3 weeks
0013		0067			0307		
0016		0083			0323		
0001		0072			0312		
0019		0093			0315		
0021	A_4	0060	B_4	4 weeks	0335	C_4	4 weeks
0022		0078			0318		
0033		0080			0320		
0030		0081			0321		
0009		0092			0333		
0017	A_5	0089	B_5	5 weeks	0329	C_5	5 weeks
0027		0068			0308		
0010		0076			0316		
0011		0086			0314		
0007		0069*			0309		
0032	A_6	0061	B_6	6 weeks	0301	C_6	6 weeks
0006		0066			0306*		
0015		0091			0331		
0005*		0074			0304		
0026		0070					
	A_{res}	0064	B_{res}	6 weeks		C_{res}	6 weeks
		0085**					

* = infection found at site of implantation
** = graft not included in section

Table 2

group D 8 animals		period of observation	group E 8 animals		period of observation	period of EHDP administration
0350 0351	D_8	8 weeks	0367 0341 0342	E_8	8 weeks	4 weeks
0352 0353 0354	D_{10}	10 weeks	0358 0368	E_{10}	10 weeks	4 weeks
0355 0356 0357	D_{12}	12 weeks	0369 0366 0348	E_{12}	12 weeks	4 weeks

Sacrificing technique

After intravenous injection of 50 mg (5000 IU) heparin, the animals were killed by intravenous injection of a lethal dose of pentobarbital sodium (Nembutal ®, S A , Abbott n v., Amsterdam) Immediately after cardiac arrest the thoraco-abdominal cavity was opened and the aorta was cannulated above the diaphragm. The plastic cannula was pushed to below the diaphragm The aorta was ligated proximal to the cannula site The inferior vena cava was opened below the diaphragm, whereupon 0 5 L physiological saline was perfused from a constant height of 100 cm The cannula was then connected to an infusion flask containing 0 5 L neutral formaldehyde (4%) The perfusion technique described took a total of about 10 minutes. Next, the graft with surrounding muscle tissue was dissected from the left lumbar region and fixed

Postmortem radiological examination

With a view to the subsequent histological procedure, the grafts were screened postmortem for the presence of mineralized bone.

Histological technique

The graft with the surrounding muscular tissue, was longitudinally divided into two halves, X and Y (cf fig 6) Part X was fixed in neutralized formaldehyde and decalcified in EDTA (10%, pH 7) and, after dehydration and embedding in paraffin, cut into sections of 7 μ (X_1, X_2 and X_3; fig 6) The

sections were stained with haematoxylin-eosin, methylgreen-pyronine, azan and, supplementally, with Alcian blue.
Part Y fixed in ethanol 70% and without decalcification was embedded in methylmethacrylate after dehydration and then cut into sections of about 110 μ with the aid of a diamond saw (instrument workshop, Department of Dentistry, University of Groningen). These sections were polished on both sides to a thickness of about 100 μ (Kent MK_2 polishing machine, Engis, Alphen aan de Rijn, The Netherlands). This applies only to grafts A_1 through A_6, B_1 through B_6 and C_1 through C_6. These sections were glued to a slide with Eukitt. From these specimens radiographs were made (X-ray apparatus Muller-80; film Kodak Spectroscopic Type 649-0) and in the case of fluorochrome labeling the sections were studied with the fluorescence microscope.

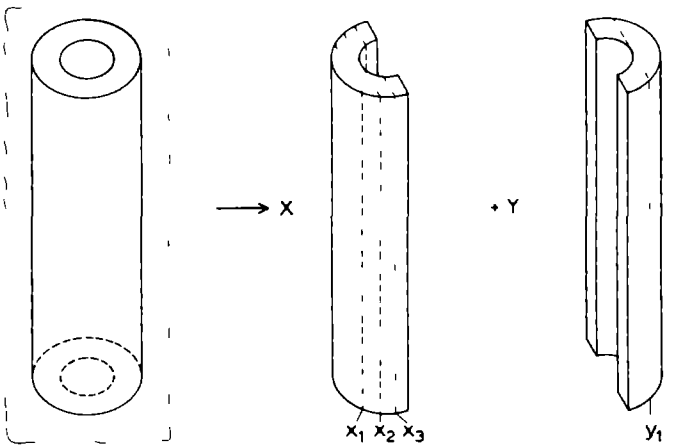


Fig 6
Diagram of the dissection procedure of the graft The graft with surrounding tissue was divided into two halves (X and Y). From part X paraffin sections were made at levels X_1, X_2 and X_3 From part Y one thick undecalcified section is ground at level Y_1 for microradiography and fluorescence microscopy

Fluorochrome staining
Because normal histological sections supply no very detailed information on osteodynamic processes such as growth and remodelling, fluorochromes were additionally used in groups A, B and C (Harris 1960; Frost 1969). Apart from tetracycline (Milch et al. 1958; Harris 1960; Harris et al. 1962), we needed another fluorochrome in this experiment in order to obtain information on the different phases of bone production (Olerud & Lorenzi 1970; Rahn

1973). Alizarin-complexon is a fluorochrome for experimental use with low system toxicity and no local effect on bone formation, which at the same time produces a readily distinguishable colour (Rahn & Perren 1972) All animals of group A, B and C which were still alive on day 19 of the experiment, were given an intravenous injection of 50 mg oxytetracycline per kg body weight (Vendarcin ®, Mycopharm, Delft) (table 3) At this time in the course of the experiment histologically quite readily identifiable bone formation has normally started (Urist et al 1967, Urist et al 1968; Urist et al. 1969)
All animals of groups A, B and C which were still alive on day 33 of the experiment, were given an intravenous injection of 30 mg Alizarin-complexon per kg body weight. At this time in the course of the experiment, the presence of complete bone formation including bone marrow has been demonstrated (Urist et al. 1967, Urist et al. 1968; Urist et al 1969) (table 3). Fragments of the animals' own femurs were also examined by the fluorescence technique as a control of the EHDP-effect on the grafts.

Table 3

fluorochrome	dose/kg	mode of administration	day of administration
Vendarcin (oxytetracycline)	50 mg	i v	19
Alizarin-complexon	30 mg	i v	33

ELECTRONMICROSCOPY

For electronmicroscopy 3 groups of animals were studied.

Group X

x_1) Two rabbits (age 5 weeks) were treated for 1 week with EHDP (5 mg/kg/day, i.m) and studied.

x_2) Three rabbits of the same age treated for 2 weeks

x_3) For each group 1 animal served as control. These animals were injected with saline for a comparable period.

The effects of EHDP were studied in the area of the cranial suturae without prior decalcification

Group Y

y_1) Three rats (Wistar, age 1 week) were treated with EHDP (5 mg/kg/day, s.c.) for 1 week and studied.

y_2) Three rats treated for 2 weeks.

y_3) For each group 2 animals served as control. These animals were only injected with saline during comparable periods.

The effects of EHDP were studied on the skull bone without prior decalcification.

Group Z

The behaviour of the inductionprocess, with a decalcified implant, was followed with and without the administration of EHDP.

z_1) Two rabbits (age 13,5 weeks) were treated for 2 weeks with EHDP (5 mg/kg/day, i.m.) and killed.

z_2) Two rabbits treated for 4 weeks after implantation and killed.

z_3) Two animals served as control and were only injected with saline for similar periods.

Small pieces of the recovered implants were studied without prior decalcification.

All animals were killed or by cervical dislocation or by intracardial injection of Nembutal. Small pieces of bone were removed from the cranial suturae of the rabbits, the skulls of the rats and from the implants in the rabbits. These fragments were transferred to a glutaraldehyde solution (2 - 5%) in phosphate buffer (pH 7,4) and fixed in the shaker for periods varying from 4 to 12 hours at 4°C. Thereafter the tissue was rinsed for 12 hours in the same buffer at the same temperature. This procedure was followed by post-fixation in osmic acid (2%) at room temperature for 1 hour, according to Palade. After dehydration in ascending series of alcohols, the tissue samples were embedded in EPON 812 (Luft 1961). In order to select the appropriate areas for electron microscopic studies, sections were cut at 0.5 µm and studied either with the phasecontrast microscope or stained with toluidine blue (2%) for light microscopy. From the blocks selected in this way, ultrathin sections were prepared (using a Reichert Om U3 microtome), placed on copper grids and contrasted with a saturated solution of uranyl acetate (Watson 1968), and subsequently with lead citrate (Reynolds 1963). The ultrathin sections were studied and photographed with a Philips EM 300 electron microscope.
On some of these sections a x-ray microprobe analysis was performed. Therefore the unstained sections, mounted on nickel grids, were analysed in a Philips EM 301 G electron microscope, equipped with the EDAX 14 mm retractable spectrometer. The voltage was 80 KeV and the probe diameters

lay within the range of 0,25 μm - 1 μm. The spot size was 32 5 nm

Summary

In a total of 125 test animals a decalcified homograft was implanted in the left lumbar region. After disqualification, 100 test animals remained for further study. They were divided into three main groups, A, B and C, each of which was subdivided at random into 6 subgroups and a few reserve animals In addition, two smaller groups D and E were formed, again subdivided into 3 subgroups and a few reserve animals.

Group A: study of the induction mechanism for 6 weeks.

Group B: study of the induction mechanism under the continuous influence of EDHP (5 mg/kg/day, intramuscularly).

Group C: study of the effect of short-term EHDP-administration on the behaviour of the induction model.

Group D: study of the behaviour of the induction model for 12 weeks.

Group E: study of the reversibility or non-reversibility of the histological changes caused by EHDP.

The grafts were studied with the aid of radiological, histological and fluorescence-microscopic techniques.

In addition the cytological effect of EHDP was studied electron-microscopically on preexisting and induced bone on a limited number of animals.

RESULTS

Introduction

This chapter describes the results obtained in terms of: a) the course of the ectopic bone induction process, and b) the effect of short-term and long-term EHDP-administration on this ectopic induction process. The description is given on the basis of paraffin sections of decalcified tissue, supplemented by fluorescence data on the presence of bone markers in non-decalcified tissue, embedded in plastic. In groups A, B and C, oxytetracycline was given on the 19th and Alizarin-complexon on the 33rd day to the animals then still alive. For the screening of implant mineralization, microradiograms were obtained. Sections of the left femoral diaphysis processed in the same way, were used as controls. Finally, the results of a supplementary electron-microscopic study of the effect of EHDP on the calvarial bone in young rabbits and rats and on the induction model described, will be presented.

Group A

In this group, which comprised 31 test animals, the induction process was studied at one-week intervals. The group served as control for the EHDP-treated groups. Examination revealed that three implants had become infected (chapter 4, table 1).

After one week the avital implant was found to be surrounded by serosanguinolent fluid containing polymorphonuclear cells, lymphocytes and phagocytotic cells. Cells of these types were found also in the original medullary cavity and in the vascular canaliculi of the implant. Incipient densification of mesenchymal cells was observed at several sites around the implant (fig. 7). The surrounding muscular tissue showed local regressive changes. The mesenchymal tissue contained incidental giant cells which were usually not in contact with the implant.

After two weeks the serosanguinolent fluid around the implant had disappeared and had been replaced by an envelope of mesenchymal cells. At some sites there was densification of these cells, which showed slightly hypertrophic features. This tissue showed a high degree of alcianophilia. As fig. 8 clearly shows, this stage was characterized by the presence of multinuclear

osteoclasts. Also phagocytotic cells and lymphocytes were present. The osteoclasts were situated against the implant and as indicated by the formation of lacunae, were involved in its resorption Capillaries accompanied by perivascular mesenchymal tissue were locally growing into these lacunae. After three weeks the features had characteristically changed There was progression of the graft resorption and of the ingrowth of capillaries In addition, however, marked osteoid formation and immature bone tissue was demonstrable in all implants (fig 9) In many cases islets of cartilage were found, especially in the vicinity of the preexisting holes in the implants The detail reproduction (fig 10) shows that, at several sites on the implant surface and in the lacunae, cells with osteoblastic features were arranged in palisades There was probably already remodelling of the newly formed bone tissue, as indicated by the osteoclasts present at these sites (fig 10) The degree of mineralization cannot be deduced from these sections but, with the radiographic technique used, mineralized bone was demonstrated in all sections. This finding corresponded with the presence of fluorescent oxytetracycline spots (oxytetracycline had been administered two days before sacrifice) (fig 11). Fluorescing rings could also be observed endosteally as well as periosteally in the femoral sections used as controls

After four weeks, the features characteristic of woven bone were evidently less marked There was active remodelling of the young bone tissue. The production of young bone tissue meanwhile continued steadily, as did resorption of the implant, much of which had already disappeared (figs. 12 and 13). The microradiograms showed an increase in the amount of mineralized bone, while the size of the fluorescent oxytetracycline spots diminished.

During the fifth week this tendency continued. The implant had disappeared to a great extent. Although in this stage there were still remnants of young bone tissue, there was an unmistakable predominance of lamellar, well-appositioned bone. Cartilage islets were no longer present. Incipient marrow formation was found between the trabeculae The implant was surrounded by a densified fibrous structure with the features of a capsule (fig 14) Oxytetracycline was still observed sporadically. The Alizarin-complexon given two days before sacrifice, however, manifested itself in readily identifiable thin lines. In the femoral sections used as controls, too, well-fluorescing Alizarin-complexon rings were present; the oxytetracycline rings in these sections had become thinner and were interrupted in some places

The transition from the fifth to the sixth week was characterized by disappearance of the trabecular bone resulting in the formation of an ossicle. The ossicle was surrounded by a capsule and contained fully developed marrow tissue, as shown in fig 15 There was continuing mineralization of lamellar bone, which had completely replaced the woven bone (fig. 16). The microra-

Fig. 7. One-week control. The decalcified implant is locally surrounded by dense *mesenchymal tissue*.

A. No. 0014. H-E 120x

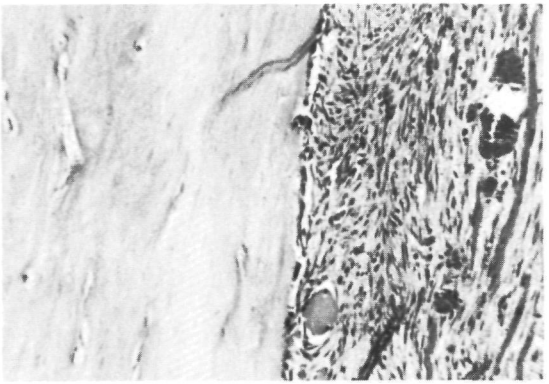


Fig. 8. Two-weeks control. There is evident resorption of the implant by osteoclasts. Capillaries are present in the lacunae. Locally, densification of alcianophilic mesenchymal tissue in contact with the implant.

A. No.0004. Alcian blue 120 x

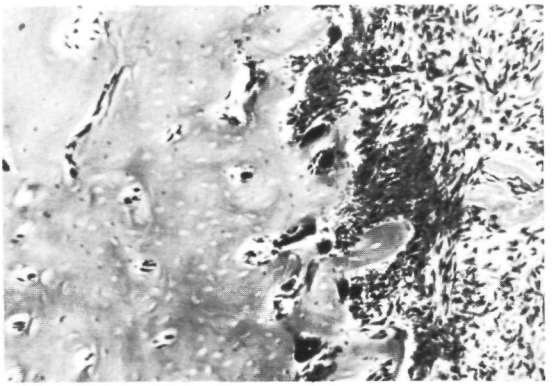


Fig. 9. Three-weeks control. Osteoid and young bone tissue is present in contact with the implant.

A. No.0013. H-E 48x

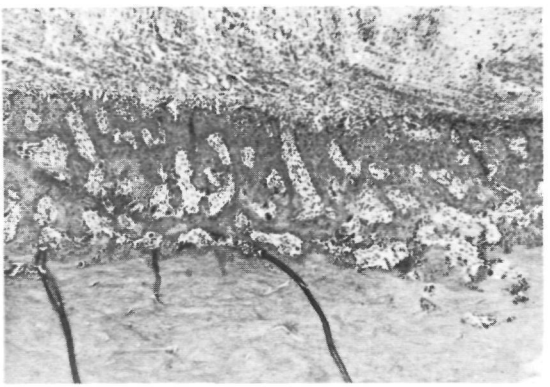


Fig. 10. Three-weeks control (detail) demonstrating remodelling of the young bone tissue. Note the osteoblasts arranged in palisades.

A.No.0013. H-E 120x

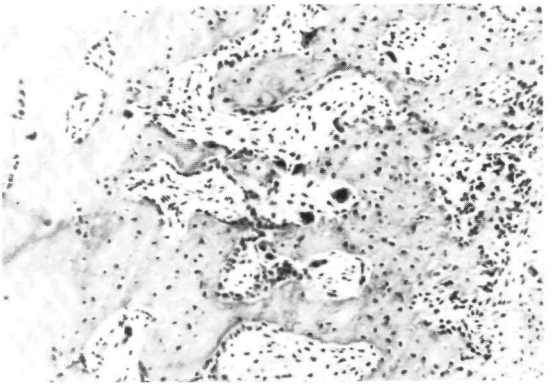


Fig. 11. Three-weeks control. This fluorescence micrograph shows oxytetracycline incorporated in the young bone tissue. Marker administered two days before sacrifice.

A. No.0013. 80x

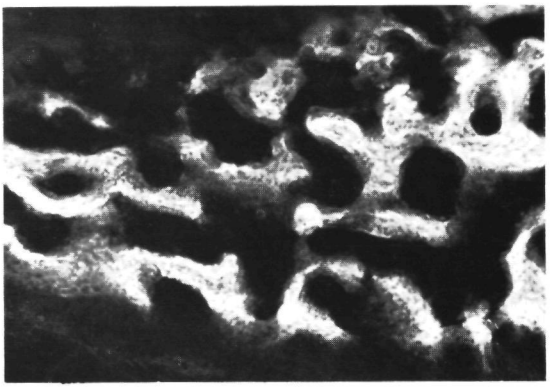


Fig. 12. Four-weeks control. A substantial part of the implant has already been resorbed. The amount of young and remodelled bone has markedly increased.

A. No.0019. H-E 48x

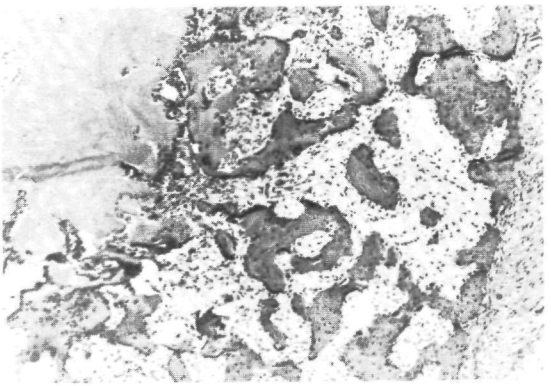


Fig. 13. Four-weeks control. Detail of fig. 12, revealing apposition of lamellar bone against remnants of young bone.

A. No.0019. H-E 120x

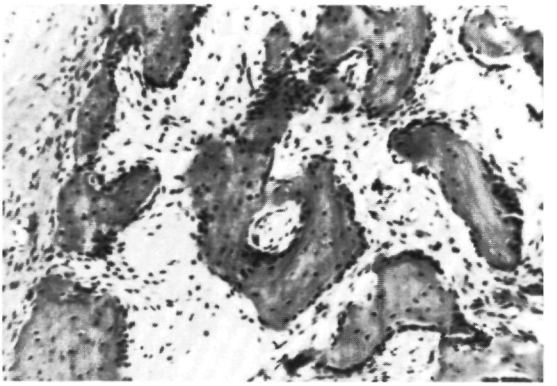


Fig. 14. Five-weeks control. The newly formed bone tissue is enveloped in a fibrous capsule. Incipient marrow formation between the trabecular bone.

A. No.0017. H-E 48x

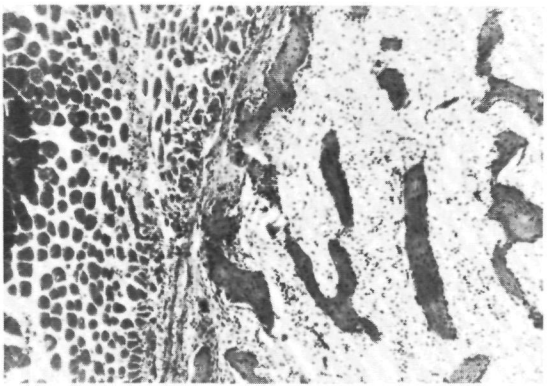


Fig. 15. Six-weeks control. An ossicle, surrounded by a fibrous capsule, has been formed, containing fully developed marrow.

A. No.0032. H-E 48x

Fig. 16. Six-weeks control. Detail of fig. 15, showing that the bone of the ossicle is of a predominantly lamellar type.

A. No.0032. H-E 120x

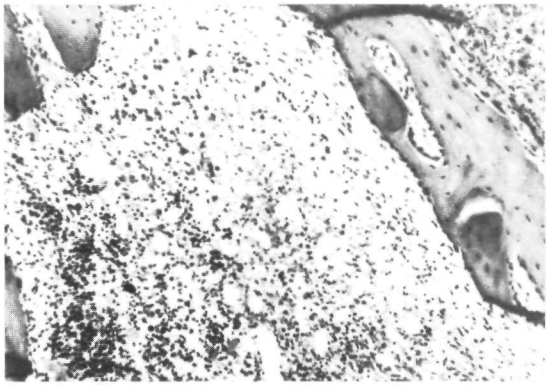


Fig. 17. Six-weeks control. Fluorescence micrograph showing the presence of the Alizarin-complexon administered 9 days before sacrifice.

A. No.0026. 80x

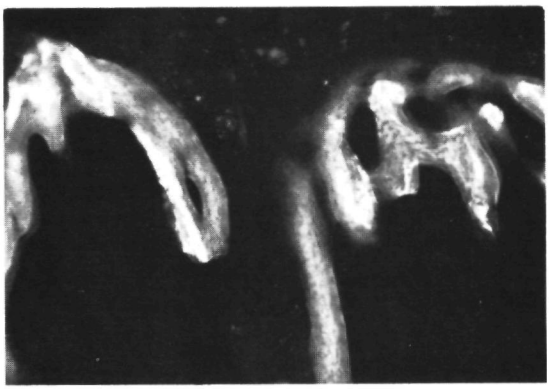
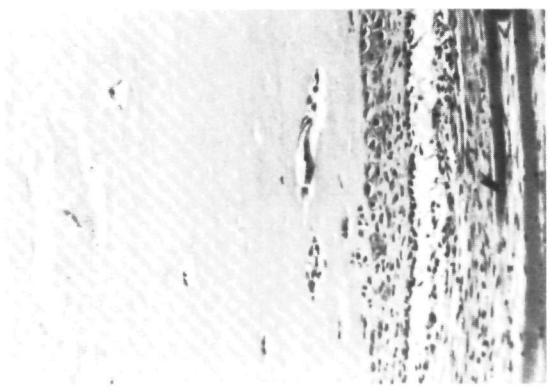


Fig. 18. Two weeks EHDP. Chondroitic tissue has been formed against the outside of the implant.

B. No.0063. H-E 120x



diograms also showed a recognizable long bone model. The findings with the bone markers were comparable with those described after five weeks' observation (fig. 17).

Summary

The induction process described showed some regularities. The process of implant resorption preceded the formation of induced bone. The newly formed young bone tissue had the typical features of woven bone. It was subject to a remodelling process, and was gradually replaced by lamellar bone. The bone with macroscopically trabecular organization disappeared, and ultimately there remained an ossicle containing fully developed marrow tissue.

In the described phases of this process, the various individual test animals showed a high degree of uniformity in findings. No bone induction was demonstrable in the three infected implants. Postmortem radiographic screening revealed mineralized bone in all implants three weeks after implantation. The microradiograms and fluorescence findings confirmed the histologically observed course of the induction process.

Group B

In this group of 34 animals, changes in the induction process in response to EHDP were studied at one-week intervals. Examination showed that five implants had become infected and one implant could not be recovered in the sections (chapter 4, table 1).

After one week there was no demonstrable difference between the results in this group and those in the control group; after two weeks, however, differences became manifest. In some implants a band of osteoid-like tissue was found, apposed to the implant. This tissue contained cartilage-like cell islets which were also found in the pre-existent lacunae of the implant (fig. 18).

After three weeks there was a marked increase of this tissue. The matrix of this tissue appeared to contain a rather small number of cells and only a few fibers. The cells showed some resemblance both to osteoblasts and to osteocytes, but could not be exactly classified (fig. 19). Another striking feature was the increase in cartilage-like fields in this tissue. As compared with the control group, the number of osteoclasts as well as the degree of resorption of the implant was less (fig. 20). At this stage, no mineralized bone was microradiographically demonstrable, and no incorporation of oxytetracycline was observed. In the femoral sections used as controls, however, fluorescing oxytetracycline rings were visible.

Although exact quantification was difficult, the prevailing impression after four weeks was that osteoclast activity in this series lagged behind that in the control group The same applies for the implant remnants. They appeared to be significantly larger than those in control animals after comparable survival periods. In comparison with the 3 weeks survivals there was a further increase in atypical tissue, including the condroitic fields (fig 21) The tissue did not seem to be subject to any remodelling (fig 22) The presence of erythrocytes, sometimes in clusters, in the matrix of osteoid-like tissue and cartilage was remarkable. There were no histological indications of any significant mineralization, for the H-E preparations lacked a 'calcium trace' The negative microradiograms were consistent with this description With Alcian blue staining, no distinct osteoid zone was distinguishable; not even at the sporadic sites where a continuous row of 'osteoblasts' was found. Another striking finding was the presence of conglomerates of polynuclear cells which were apparently unrelated to the implant These cells showed some very unusual features: marked vacuolization and sometimes cell deformation (fig 23) The detail shown in fig. 24 illustrates the atypical character of the tissue cells

The above described features were still present after five weeks, although the impression was that the chondroitic fields had somewhat diminished in size The incipient long bone formation observed in the controls, remained absent. At some sites, however, incipient marrow formation was observed in an otherwise rarefied reticular tissue (fig. 25) The Alizarin-complexon administered two days before killing, was not traceable in the implant sections Nor was it found in the femoral sections used as controls.

The situation after six weeks EHDP-administration differed little from those after five weeks. Again there was a further decrease of the chondroitic tissue. To illustrate this, we refer to fig. 26 (A, B, C, D). As compared with the controls, the striking finding was that no ossicle had formed and that the marrow tissue present was less pronounced

Summary

Although exact quantification could not be performed, resorption of the implant in this group unmistakable lagged behind that in control animals. This was manifest in a diminished presence of osteoclasts in contact with the implant, of which a large part was still present after six weeks. There were local conglomerates of polynuclear, highly vacuolated cells which were not in direct contact with the implant. At a fairly early stage an osteoid-like tissue was formed, which subsequently extended to fields of a very irregular shape The tissue was poor in cells and fibers as compared with that in controls. Moreover, large fields of chondroitic tissue were found, which seemed to

Fig. 19. Three weeks EHDP. Irregular osteoid-like tissue has been formed which contains cartilage islands.

B. No.0067. H-E 120x

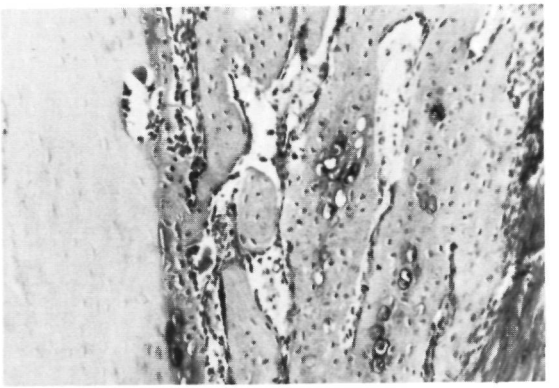


Fig. 20. Three weeks EHDP. A field of chondroitic tissue lies against the outside of the implant. As compared with the control group (fig. 9) there is strikingly little osteoclast activity.

B. No.0084. H-E 120x

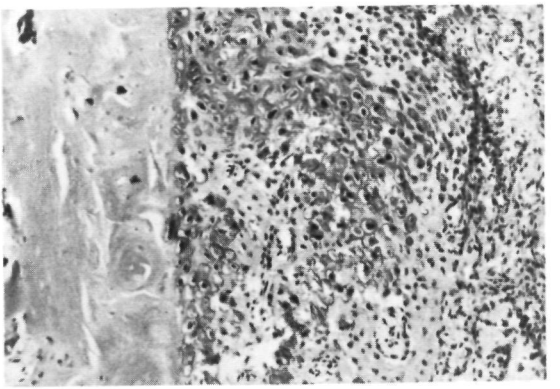


Fig. 21. Four weeks EHDP. Predominance of tissue resembling osteoid tissue containing cartilage structures.

B. No. 0078. H-E 48x

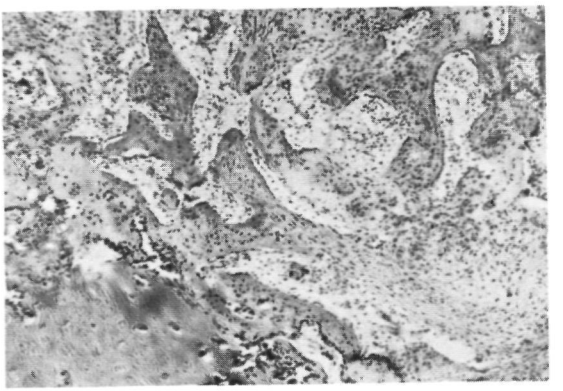


Fig. 22. Four weeks EHDP. Detail showing the absence of signs of remodelling. The irregular tissue contains scattered osteocytes. The interstitial substance has an 'aqueous' aspect. Note the absence of palisades as compared with the control group (fig. 10).

B. No.0072. H-E 120x

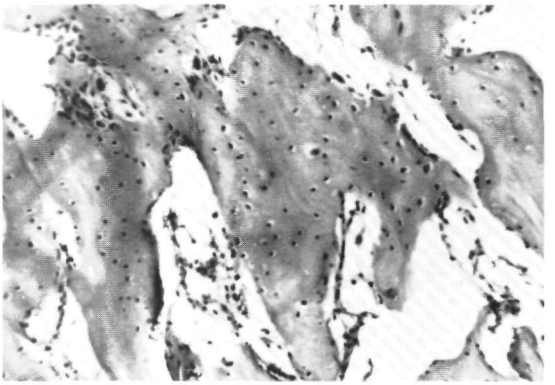


Fig. 23. Four weeks EHDP. Detail showing conglomerate of atypical polynuclear cells.

B. No.0072. H-E 120x

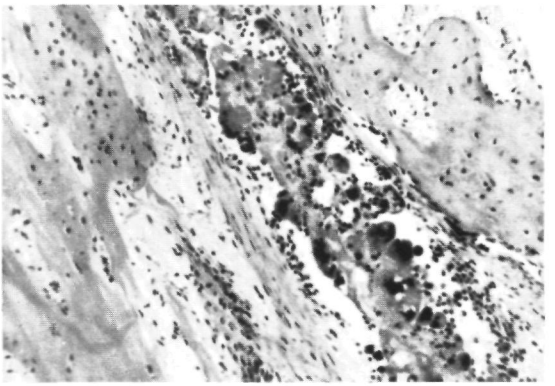


Fig. 24. Four weeks EHDP. In this micrograph the atypical character of the 'bone cells' is clearly visible.

B. No.0072. H-E 300x

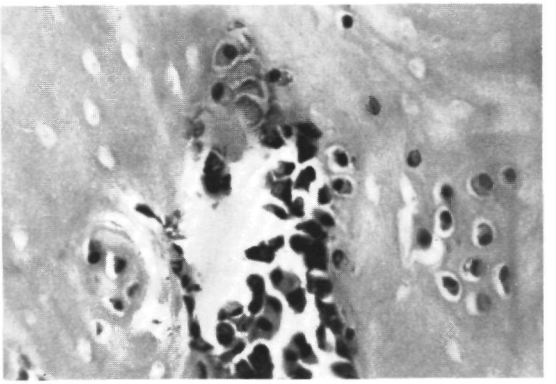


Fig. 25. Five weeks EHDP. This micrograph shows the persistence of atypical bone tissue and incipient marrow formation. A substantial implant remnant is still visible.

B. No.0068. H-E 120x

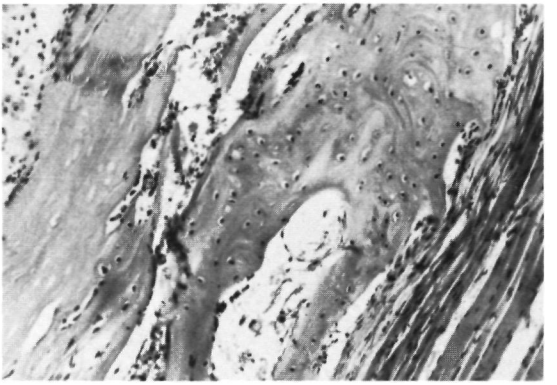
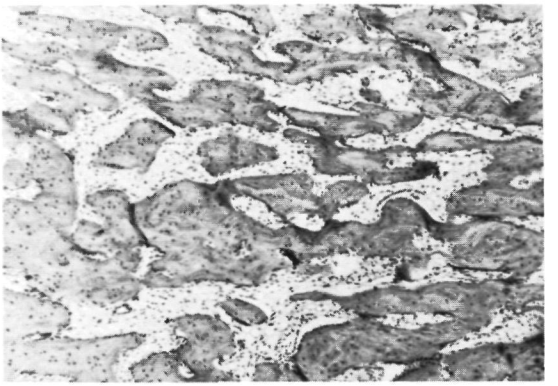
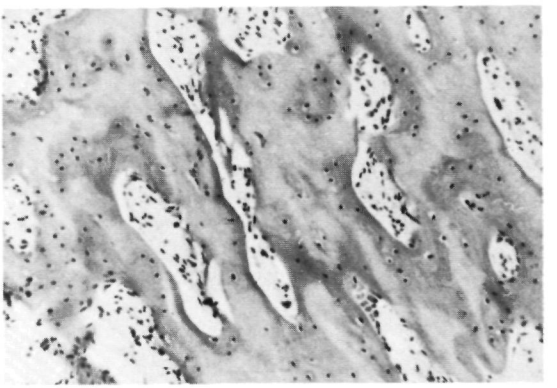


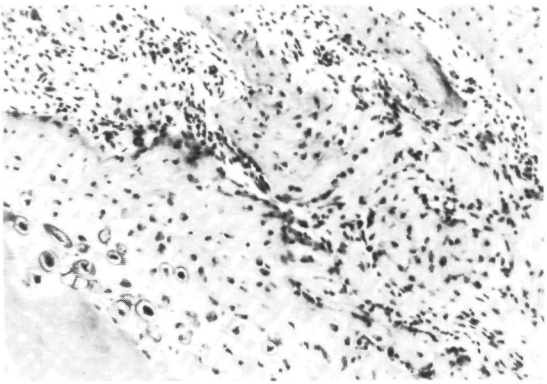
Fig. 26. Six weeks EHDP. No ossicle has formed as compared with the control group (fig. 15). The central space remains filled with trabecular structures interspersed with marrow tissue (A). The tissue retains its atypical structure (B). Continued presence of substantial implant remnants, chondroitic structures (C) and the unusual conglomerates of polynuclear cells (D).
(A) B. No.0074. H-E 48x



(B) B. No.0064. H-E 120x



(C) B. No.0074. H-E 120x



(D) B. No.0074. H-E 300x

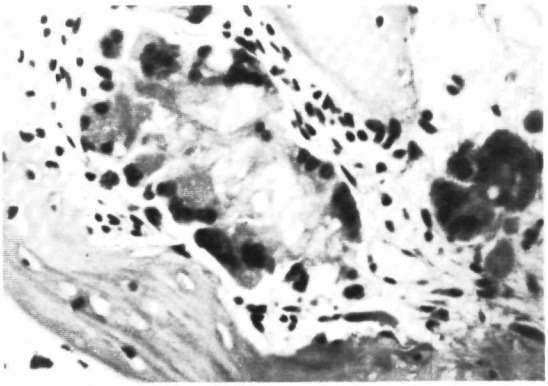


Fig. 27 One week with, five weeks without EHDP. Survey and detail of ossicle formed. As compared with the control group (fig.16) the bone is less mature and more trabeculae are present in the marrow cavity (A). Detail (B) shows remnants of the dead implant with lamellar bone in apposition.

(A) C. No.0310. H-E 48x

have decreased in size after 5 - 6 weeks The cells in the osteoid-like tissue showed atypical features and could not be properly identified With the techniques used, no mineralization of any significance was demonstrable in the implants The osteoid-like tissue, moreover, did not seem to be subject to remodelling The formation of an ossicle in fact failed to occur, but normal marrow formation was observed during the final weeks of the experiment In this group, too, the findings of postmortem radiological screening, the microradiograms and the fluorescence findings correlated with the histological findings The oxytetracycline administered was not traceable in the implant sections It was only found in the femoral sections used as controls No induction was demonstrable in the five infected implants in this group

Group C

The 30 animals in this group received EHDP for 1, 2, 3, 4, 5 and 6 weeks after implantation, respectively. All were sacrificed after six weeks. Three implants of this series had become infected (chapter 4, table 1).

EHDP-administration for one week resulted in the formation of an ossicle containing marrow tissue of normal appearance. In comparison with the control implants, the bone of the ossicle was less mature Remnants of the implant and cartilageous tissue were still observed. In the marrow space a few trabeculae were present (fig 27A, B) In addition to normal osteoclasts, there were sporadic polynuclear vacuolated cells as found in group B. The microradiograms confirmed the presence of mineralized bone. Only sporadic traces of the oxytetracycline administered were observed, whereas the Alizarin-complexon was clearly visible in well-defined thin lines.

After two weeks of EHDP-administration the long bone model was hardly recognizable It had a trabecular structure interspersed with otherwise normal marrow tissue A large part of the implant was still present (fig 28A). In addition to young bone tissue there was an increase in osteoid-like tissue and chondroitic structures, as compared with the one week treated animals. There was some remodelling, but formation of lamellar bone was rare (fig. 28B). All microradiograms showed mineralized bone in this group also. The oxytetracycline administered was not traceable, however, and the Alizarin-complexon was manifest more in spots than in lines.

After three weeks EHDP-administration, the long bone model was no longer recognizable Locally there was still a fragment of young bone tissue, but there was no longer any trace of lamellar bone. The sections stained with Alcian blue showed no clearly distinguishable osteoid zones; however, there was a large amount of osteoid-like tissue and of chondroitic structures interspersed with normal marrow tissue, as found in group B (fig. 29A). Again there was a substantial implant remnant with few osteoclasts and, at some

distance, the peculiar polynuclear cells The tissue again contained the atypical cells (fig 29C), and showed gradual transitions to tissue with chondroitic cells (fig 29B) Only a few of the microradiograms were positive The Alizarin-complexon given 12 days after discontinuation of EHDP, was traced in only one implant

The findings after four weeks of EHDP-administration showed a marked resemblance to those obtained in group B after five and six weeks There was a further increase in osteoid-like tissue, irregularly shaped with normally formed marrow tissue in the spaces between (fig. 30 A,B). As compared with the features in group B after four weeks continuous EHDP-administration, the fields of the chondroitic tissue were less numerous and less extensive

There was a substantial implant remnant with only scattered osteoclasts. In view of the absence of a 'calcium trace' in the H-E preparations and the negative microradiograms it was exceedingly improbable that any significant mineralization had taken place Fluorescing Alizarin-complexon was observed in none of the implants The control femur sections, however, showed an occasional trace of this fluorescing substance

After five weeks of EHDP-administration the similarity to the longer survivors in group B was evident. There were no clearly demonstrable differences in the histological sections and this was confirmed by the microradiograms and the fluorescence microscopic findings

Summary

Apart from some slight differences the results obtained after one week of EHDP-administration, showed marked resemblance to the control animals of group A An increasing persistence of the trabecular bone was found after 2 weeks EHDP After 3 weeks of EHDP-administration the osteoid-like fields described in group B, remained present, although a slight mineralization was occasionally observed in these implants Apart from the presence of normal bone marrow there was no longer any agreement with the findings in the six-week survivals of the control group The animals treated for more than 3 weeks showed a very close resemblance to those in group B. Postmortem radiographic screening of implants and the microradiograms confirmed that the turning-point for recognizable mineralization was at three weeks of EHDP-administration. Comparable observations were done with oxytetracycline and Alizarin-complexon

Group D

In this series the behaviour of the implant was studied through 12 weeks after implantation. In comparison to the 6 weeks survival of the control group no

essential change in the structure of the bone was found to occur up to 12 weeks (fig. 31A, B).
Although quantification is difficult, the impression was that the volume of the ossicle had slightly decreased after these 12 weeks. Moreover in the medullary cavity an unmistakable increase in the amount of degenerating material was observed in the course of time.

Group E
A mineralization was radiologically identifiable 4 weeks after discontinuation of EHDP-administration. Two weeks later the degree of mineralization was found to have increased, not only in the lamellar but also in the trabecular bone. In the histological sections four weeks after discontinuation of EHDP-administration, distinct remodelling was observed. This was manifested by, among other things, a palisade arrangement of the osteoblasts, especially at the periphery (fig. 32). This trend continued during the subsequent weeks, and part of the trabecular bone also became involved. Nevertheless a fair part of the medullary cavity remained filled with these structures throughout the period of observation (fig. 33A, B). The atypical tissue described in group B was also found in this group, although the amount decidedly decreased in the course of time. The persistence of implant remnants was again a striking feature in comparison with group D (fig. 33A, B).

Fig. 27.
(B) C. No.0302. H-E 120x

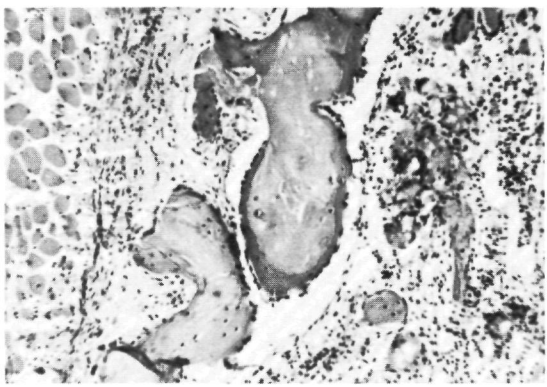
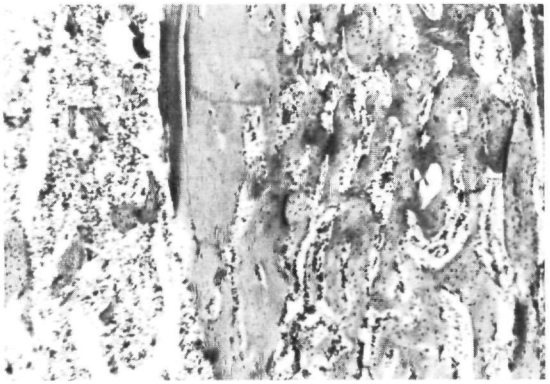


Fig. 28. Two weeks with, four weeks without EHDP. As compared with the control group (fig. 16) a large implant remnant is still present (A). In the marrow cavity many trabeculae consisting of immature bone tissue are present (B).

(A) C. No.0305. H-E 48x



(B) C. No.0334. H-E 48x

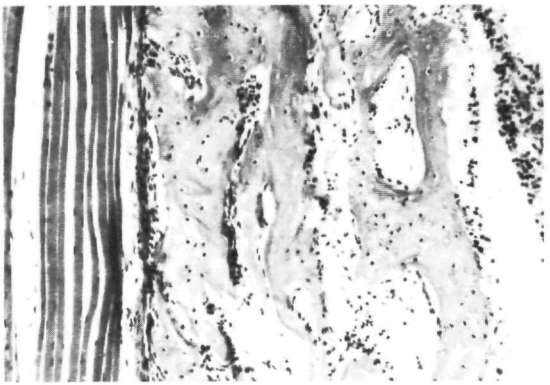
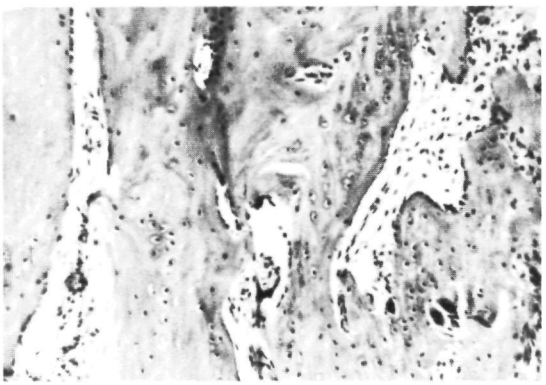
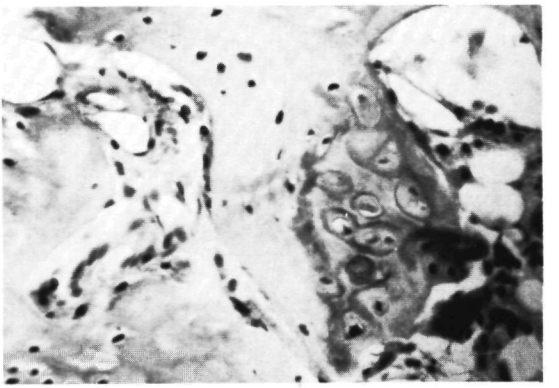


Fig. 29. Three weeks with, three weeks without EHDP. There is close similarity to those implants treated for six weeks. Tissue of irregular form, showing little remodelling (A). Details show cartilage islands (B) and 'atypical osteocytes' (C).

(A) C. No.0324. H-E 120x



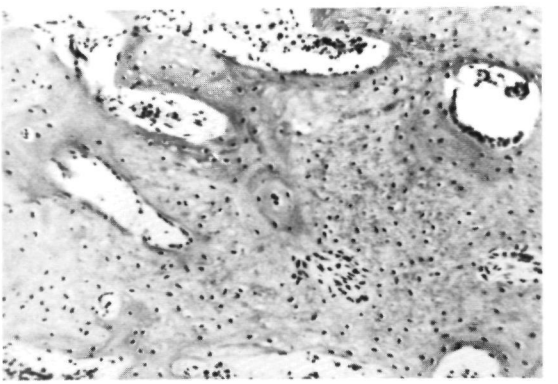
(B) C. No.0323. H-E 300x



(C) C. No.0324. H-E 300x

Fig. 30. Four weeks with, two weeks without EHDP. The tissue shows a striking similarity with that after six weeks of EHDP (fig. 26B). Very atypical tissue (A). Large implant remnant. Marrow tissue in between 'trabecular' tissue (B).

(A) C. No.0318. H-E 120x



(B) C. No.0312. H-E 120x

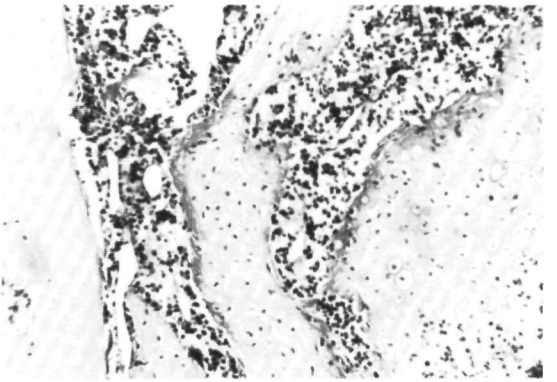
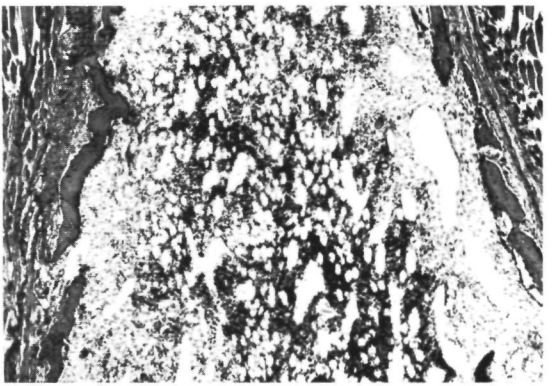


fig. 31. The ossicle 10 weeks after implantation. The ossicle has a normal shape (A). It consists of lamellar bone, lined with osteoblasts on the medullary side (B).

(A) D. No.0354. H-E 48x



(B) D. No.0352. H-E 120x

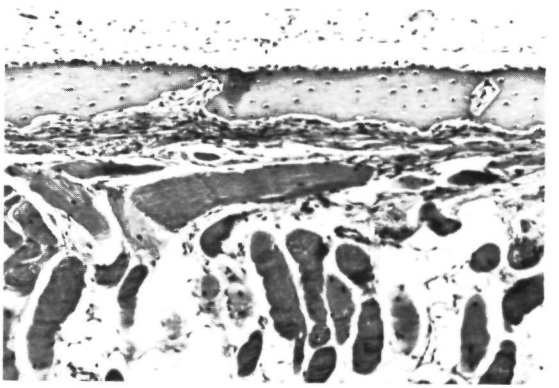


Fig. 32. Micrograph of part of the ossicle 4 weeks after withdrawal of EHDP. Partial remodelling at the periphery. Note the irregular trabecular structures in the medullary cavity.

E. No. 0367. H-E 120x

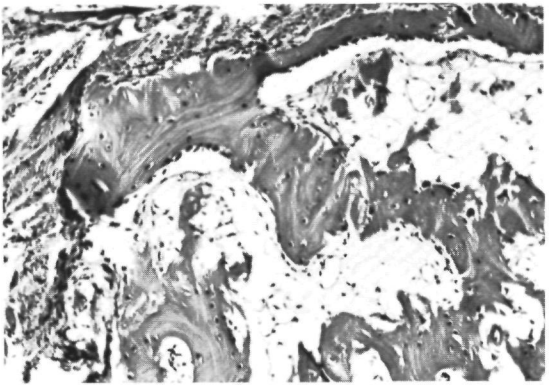
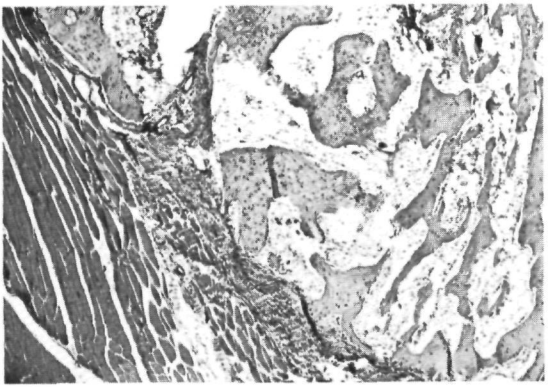
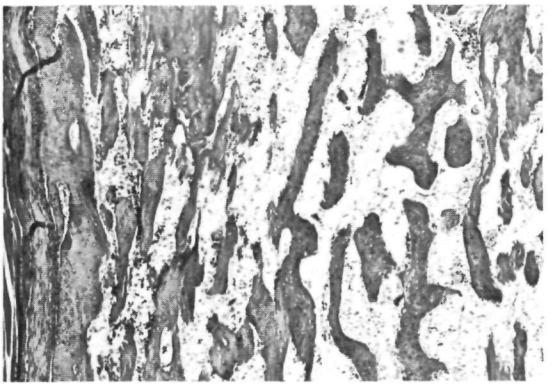


Fig. 33. General view of the ossicle 6 weeks after withdrawal of EHDP. Locally well-shaped lamellar bone visible on the outside (A, B). The medullary cavity contains an implant remnant as well as partly remodelled bone and various transitional forms of the atypical tissue described in group B (B).

(A) E. No.0358. H-E 48x



(B) E. No.0358. H-E 48x



ELECTRONMICROSCOPY

The results of a preliminary study on the ultrastructural effect of EHDP on existing bone and on the process of induced bone formation are described (special attention was paid to the osteoblasts)

Group X *(cranial suturae of young rabbits)*
The osteoblasts of the control animals exhibited a well developed, slightly dilated RER The markedly extended Golgi areas contained an amorphous or a filamentous substance and the surrounding cytoplasm showed small electrondense filaments. The mitochondria showed no unusual features.
The RER and Golgi areas of the osteoblasts in the EHDP-treated animals were comparable to those of the controls. However, the structure of the mitochondria appeared to be strikingly changed. After 1 week EHDP-treatment the mitochondria showed either a somewhat foamy, light matrix, containing granules, or a dark matrix, containing a large number (5-20) of crystalloid granules (fig. *1, 2*) After 2 weeks EHDP-treatment the number of mitochondria had decreased and they appeared to contain fewer and more scattered granules (fig *3, 4*)

Group Y *(calvaria of young rats)*
Depending on the location and activity of the control bone sample, the osteoblasts revealed a more or less strongly dilated RER The Golgi areas may be dilated and there were some fat droplets and lysosomes in the cytoplasm The mitochondria had a normal aspect Generally there was a combined occurrence of a strongly dilated RER, dilatation of Golgi areas and numerous lysosomes
After 1 week treatment with EHDP the dilatation of the RER and Golgi areas in many osteoblasts was more pronounced. Especially striking were the mitochondria, most of which contained crystalloid granules in their matrix. However, some osteoblasts did not exhibit any unusual features, apart from the dilatation of the RER (fig *5, 6*). Comparable observations were made after two weeks EHDP-treatment, although the number of crystalloid granules had decreased (fig. *7, 8*). With the EDAX-analysis it appeared that the crystalloid granules in the mitochondria of the treated groups contained calcium
The aspect of the osteocytes in both groups (X and Y) differed with location. Almost all of the control as well as the EHDP-treated animals, exhibited dilated RER, well developed Golgi areas, some lysosomes and fat droplets. Occasionally these cells are surrounded by a dark zone of condensed foci containing calcium (EDAX-analysis) At a variable distance from the cell

deposits of hydroxyapatite were found.
No evident difference in the character of the intercellular ground substance was found between the control and EHDP-treated animals in both groups. The area near the osteoblasts contained only scattered foci of mineralization to a variable extent. Areas at a greater distance appeared to contain interwoven collagen fibers and larger foci of mineralization, with, however, locally differing concentration.

Summary
The effect of EHDP on the osteoblast-mitochondria is quite striking. After 1 week EHDP-administration numerous granules in the matrix of most mitochondria were present. After 2 weeks the number of these matrix granules had diminished and most mitochondria had a normal aspect. No difference in the aspect of Golgi areas was found between experimental and control animals.

Group Z *(induced ectopic bone)*
Apart from the degree of mineralization and the number of osteocytes, the control implants showed no significant difference in the 2 and 4 weeks survivals.
The osteoblasts with their distinct nuclei and nucleoli were characterized by an RER-aspect which varied from very markedly dilated anastomosing cisternae to distended fragmentations of the endoplasmic reticulum. The Golgi areas were often arranged in groups, and the lamellae were either flattened or broadened. In the latter case there were, in their immediate vicinity or more peripherally, characteristically shaped structures, sometimes called 'spherical portions' (containing a finely granulated mass), beside the 'cylindrical portions' (rectangular ø 90 - 140 mμ, length 360 - 650 mμ) and the 'secretory granules' (fig. *10*).The latter two are filled with fine parallel filaments believed to represent procollagen. The abovementioned elements originate from the Golgi apparatus. Beside normal mitochondria, small and large lysosomes were often present. Glycogen areas as well as occasional cilia were found. The adjacent pre-bone matrix comprised varying numbers of nucleation points, which gradually increased in number further away from the osteoblasts and showed the normal features of extracellular mineralization of young bone tissue (fig. *9*).
The osteocytes had some long RER-cisternae and a small Golgi area beside lysosomes of varying size (fig. *11*).
After 2 and 4 weeks of EHDP-administration, the implant tissue showed a heterogeneous aspect; beside regular formation of osteoid-like tissue, local cartilage was found in which the aspect of the chondrocytes varied from

normal to hypertrophic or even degenerated
The osteoblasts had the same characteristics as those in the control implants (fig. *12*), showing fragmentations as well as dilated anastomosing RER-cisternae. The Golgi lamellae found in juxtanuclear as well as in peripheral positions, were generally flattened Again, the spherical and cylindrical portions, described above, were found in their vicinity In addition to an occasional lipid droplet, delicate cytoplasmic filaments and an occasional cilium were found There were several irregularly shaped lysosomes of different size, sometimes with characteristics of autophagy Generally the mitochondria seemed normal, but in the 2-weeks-old implants some crystalline matrix inclusions were occasionally found No mineralization was observed in the adjacent pre-bone matrix, not even in the form of foci. The matrix consisted of densely interwoven collagen fibrillae of varying size and length, which at several sites showed an abnormal arrangement: the bundles of collagen fibrillae were arranged in alternate directions Apart from split fibrillae, numerous shredded ends were localized which in cross section proved to show irregular rosette-like structures (fig. *14*) No condensed foci, containing calcium, could be detected with the EDAX-analysis.
A typical feature was the presence of many free erythrocytes in the osteoid-like tissue and in the matrix of the cartilage.
The osteocytes, localized deeper in the non-mineralized osteoid-like tissue, had nuclei with distinct nucleoli and several cytoplasmic processes (fig *13*) The RER-features varied from dilatated cisternae to fragmentations There were several Golgi areas, preferably arranged in groups, which were either markedly dilatated or consisted of flat lamellae The features therefore differed from those of the osteocytes in the untreated implants In addition to a few lysosomes, glycogen areas were also present

Summary

The bone tissue found in the untreated implants showed normal electron-microscopic features, both of the cell constituent and of the interstitial substance The tissue in the EHDP-treated implants was evidently different Although there was no distinct osteoblastic EHDP-effect, the osteocytes were abnormal They resembled slightly less active osteoblasts and will be referred to as 'atypical osteocytes' The chondrocytes also sometimes showed hypertrophic features and occasional degeneration. The interstitial substance in the osteoid-like tissue also showed an atypical aspect The collagen fibrillae often presented an abnormal appearance and a distinctly alternating course. Several free erythrocytes were encountered in the osteoid-like tissue and in the chondral interstitial substance. There was no evidence of mineralization; not even in the form of foci

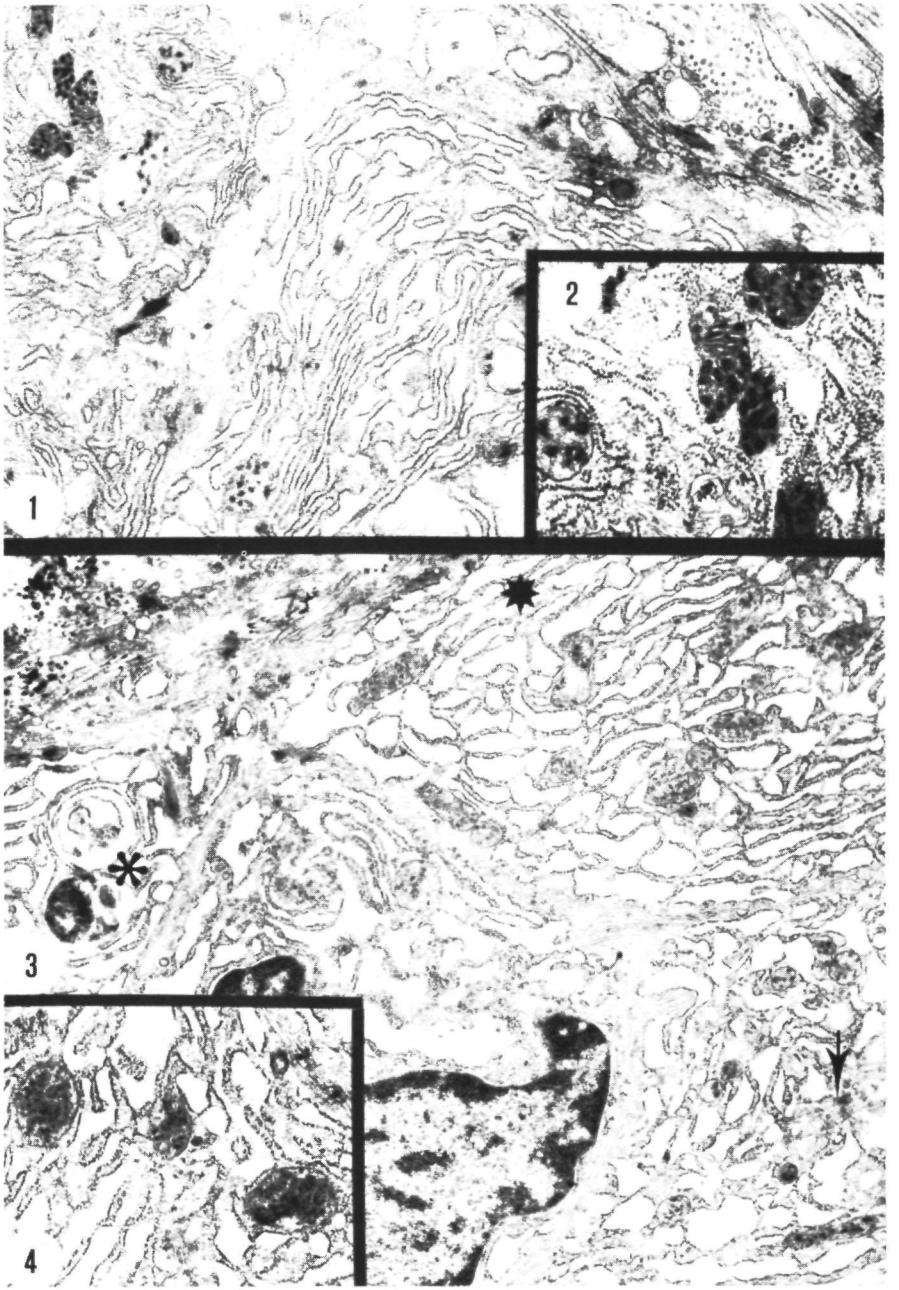

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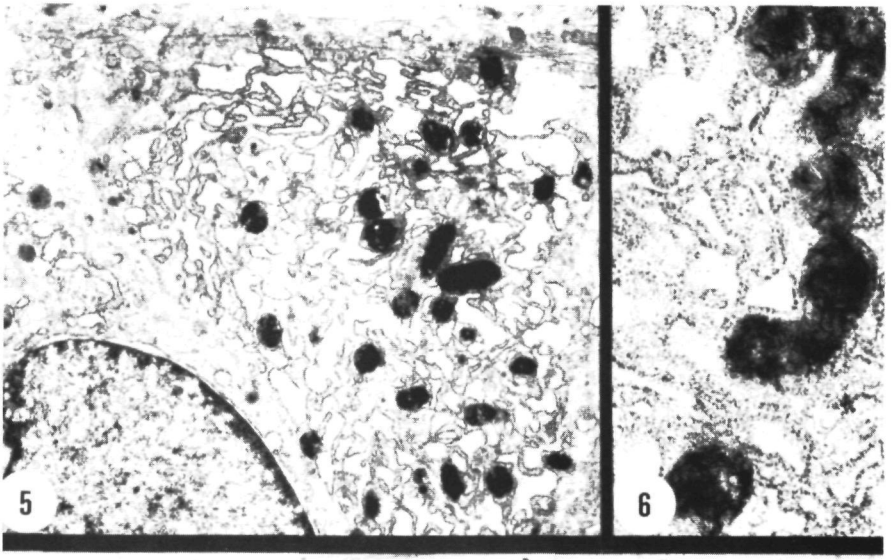
Fig 1 Group X after treatment with EHDP for 1 week (rabbit)
Notice the inclusions in the mitochondria which lie between the dilated cisternae of RFR of two osteoblasts On upper right the extracellular matrix with electron-dark precipitates of calcium on and between collagen fibrils is clearly visible
Magnification 13 000 ×

Fig 2 Detail of figure 1
The numerous crystalloid-granular inclusions are clearly defined in the matrix of the mitochondria
Magnification 23 000 ×

Fig 3 Group X after treatment with EHDP for 2 weeks (rabbit)
The osteoblasts exhibit a well developed dilated RER (➧) near some Golgi areas (↗) and autophagous vacuoles (✱) The mitochondria are conspicuous by their fine inclusions On upper left, part of an adjacent portion of matrix with foci of mineralization
Magnification 9 000 ×

Fig 4 Detail from the same group as in fig 3
Intramitochondrial inclusions are clearly smaller and less well defined than after 1 week EHDP
Magnification 14 000 ×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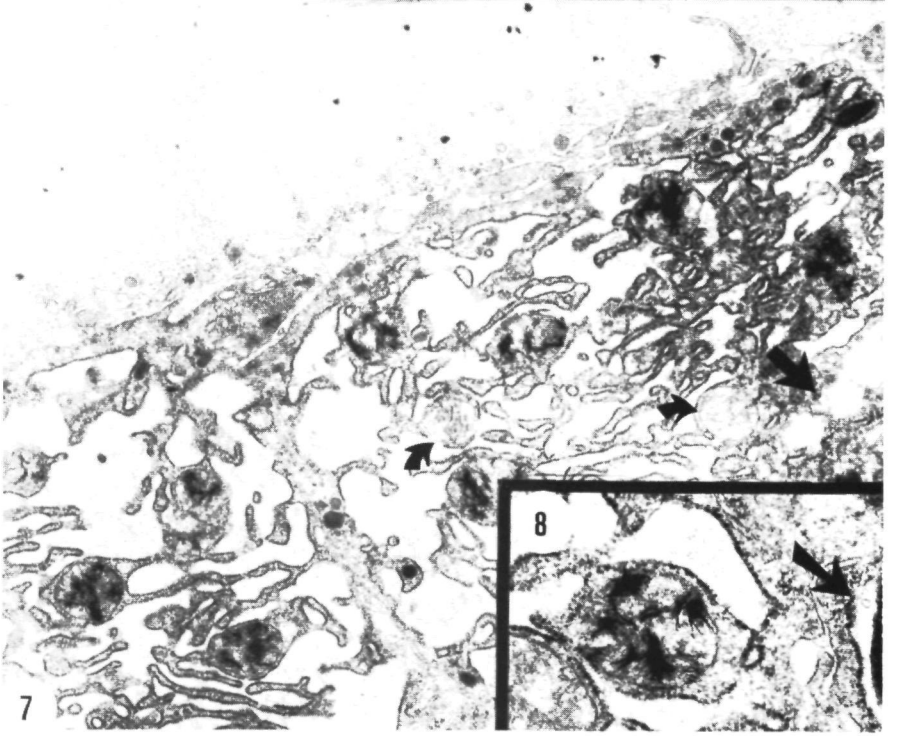
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Fig 5 Group Y after 1 week treatment with EHDP (rat)
Mitochondria with conspicuous inclusions situated between the dilated RER of an osteoblast
The adjacent matrix exhibits several foci of mineralization
Magnification 7 000 ×

Fig 6 Detail from same group as in fig 5
Dilatation of the RER is clearly demonstrated, it is evident that almost all the intercristal spaces of the mitochondria are filled with needle-shaped crystals, masking the mitochondrial structure
EDAX-analysis showed that the inclusions consist largely of calcium
Magnification 26 000 ×

Fig 7 Group Y after 2 week's treatment with EHDP (rat)
The two osteoblasts shown exhibit grossly dilated RER and a dilated Golgi area (➘) Several mitochondria contain conspicuous crystalline inclusions, while others appear free of inclusions (➘) At the top, matrix with cross sections of collagen fibrils containing foci of mineralization
Magnification 10 500 ×

Fig 8 Detail from same group as in fig 7
A clear reduction of the still needle-shaped, aggregated crystalline inclusions in the mitochondria, in comparison with 1 week EHDP-treatment Next to it, part of a mitochondrion free of inclusions
➘ = nucleus and nuclear membrane
Magnification 26 000 ×

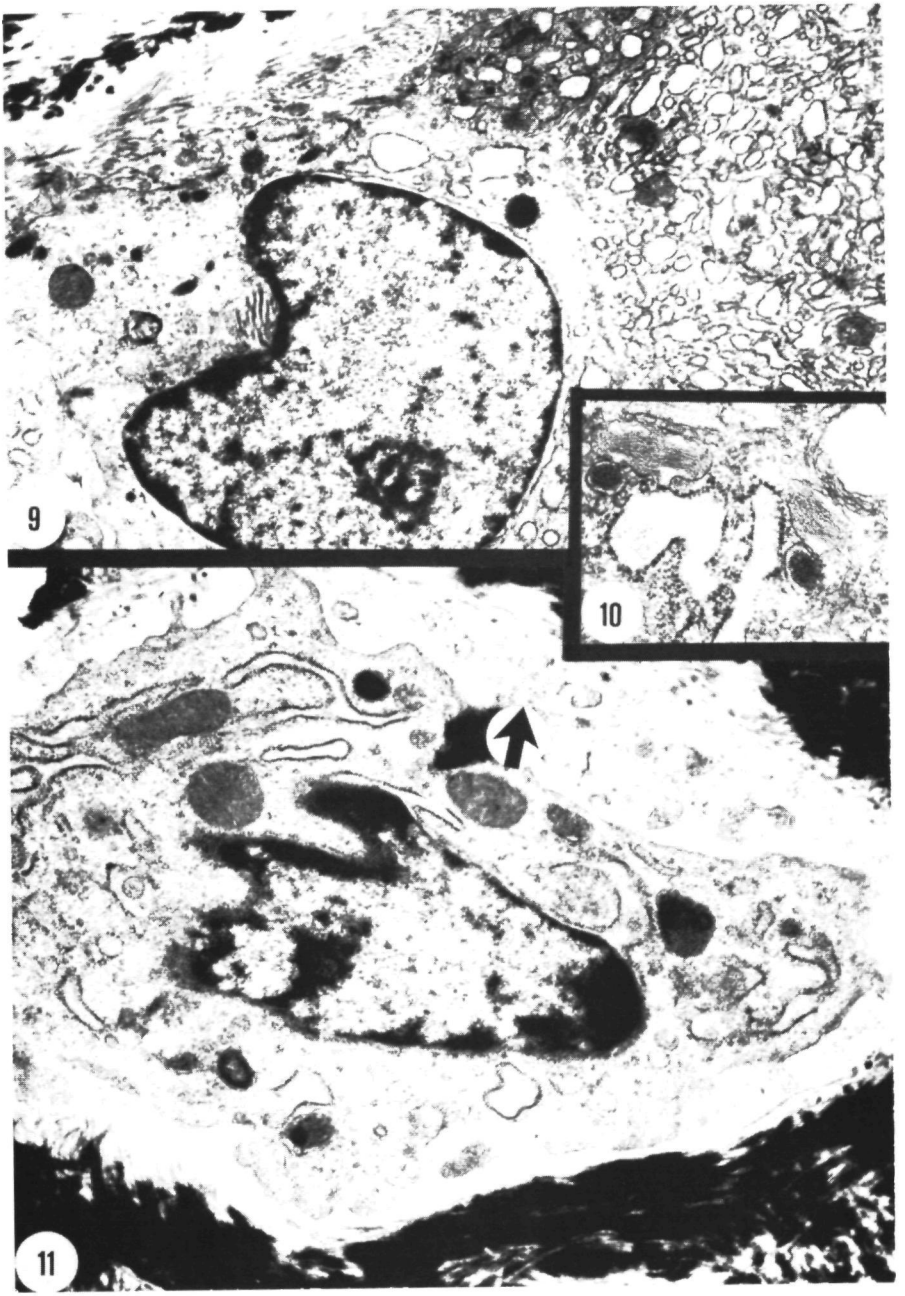
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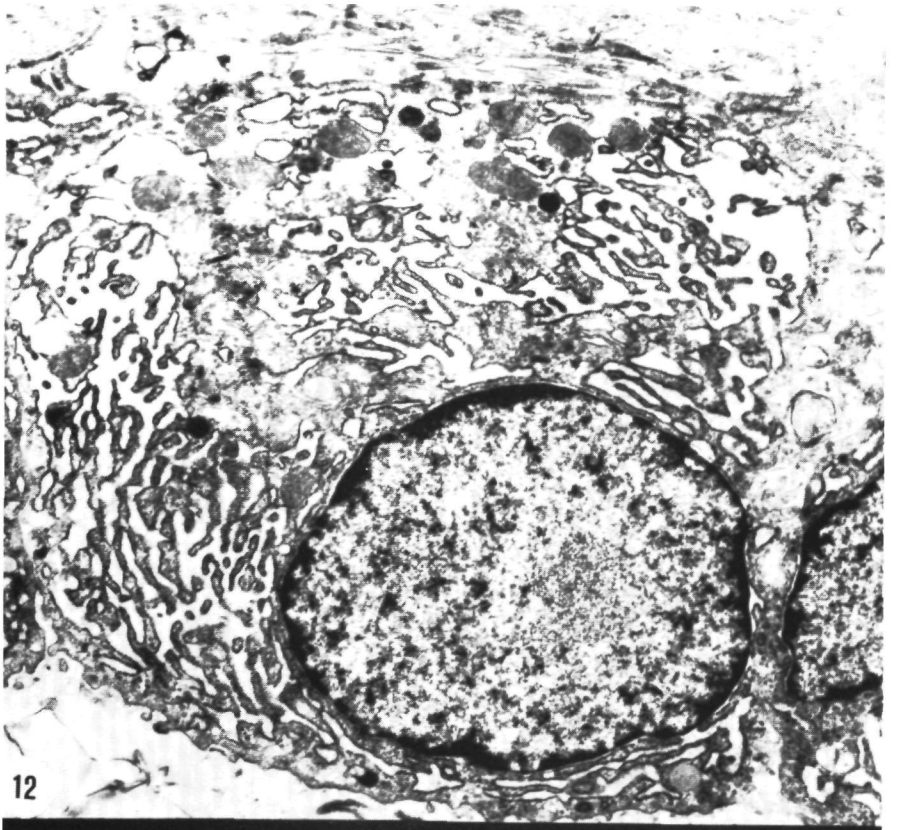
Fig 9 Group Z control 2 weeks after implantation (rabbit)
The osteoblasts exhibit varying aspects, on the right, much fragmented RER, little RER and some lysosomes on the left The immediately adjacent matrix of the bone does not yet contain any foci of mineralization, but the following area clearly exhibits the electron-dark masses of such foci (the light patches in between are artifacts of the method of preparation without decalcification which gives a different contrast of the varying structures)
Magnification 10 500 ×

Fig 10 Detail of the same group as in fig 9
The activity of the osteoblasts is reflected in the active aspects of the Golgi areas which exhibit spherical and cylindrical portions and secretory granules
Magnification 29 000 ×

Fig 11 Group Z control 4 weeks after implantation (rabbit)
An osteocyte in the middle of an area of mineralization The cytoplasm contains some sparsely distributed RER some mitochondria and lysosomes Cell debris is often found in the pericellular area due to artifacts of manipulation of bone cut without prior decalcification (➧)
Magnification 16 000 ×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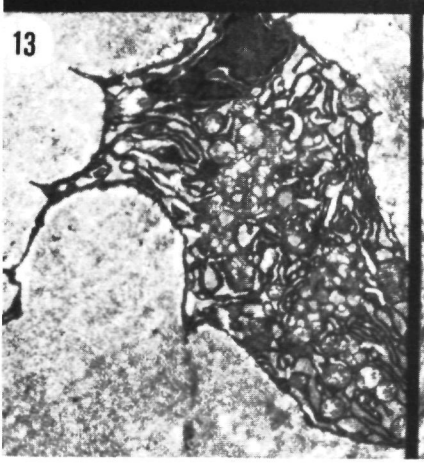
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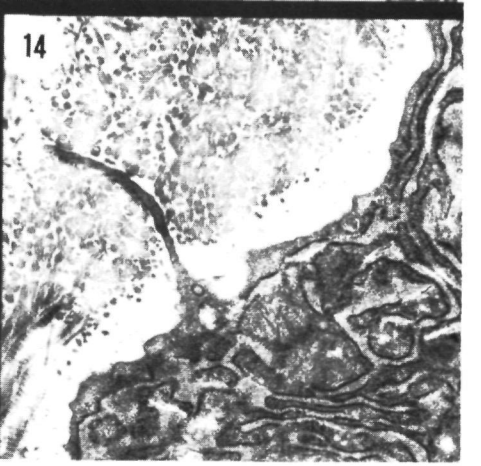
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Fig. 12. Group Z: after treatment with EHDP for 4 weeks (implantation, rabbit).
This osteoblast has a vacuolar apparatus and dilated, anastomosing RER as well as other organelles, indicative of functional activity. The adjacent matrix shows some cytoplasmic extensions and some cellular debris, but no evidence of mineralization.
Magnification: 9.000 ×

Fig. 13. Group Z: after 2 weeks with EHDP (implantation, rabbit).
This low magnification of an osteocyte demonstrates clearly abnormal cytoplasm, conspicuous and ballooning mitochondria, light Golgi areas and long dilated, anastomosing cisternae of RER that almost fill the entire cytoplasm, displacing the somewhat pycnotic nucleus. Surrounding the osteocyte one observes vast quantities of bundles of collagen fibrils interspersed with cytoplasmic extensions and some electron-dark debris. EDAX-analysis of this area was negative for calcium.
Magnification: 3.900 ×

Fig. 14. Group Z: after 4 weeks with EHDP (implantation, rabbit).
Detail of an osteocyte with cytoplasmic extension and pericellular area with matrix. The portion of the cell shown appears active. The adjacent matrix contains some collagen fibrils of unusual consistency.
Below, left: longitudinally cut fibrils with ragged ends.
Above: cross-sections of the collagen fibrils exhibit irregular contours and varying diameters.
Magnification: 14.000 ×

DISCUSSION

The results described in the preceding chapter clearly indicate that avital homologous bone, decalcified according to the method described by Urist, is capable of inducing bone. This provides us with a readily reproducible model of ectopic osteoneogenesis, which seems to meet the requirements for a study of the influence of EHDP on various aspects of ectopic bone formation. In the experiment described in this thesis, positive induction was observed in 100% of the control and EHDP-treated animals; the first signs of positive induction in the rabbits as a rule occurred in the course of the third week. Our findings are in agreement with the percentage of 95 - 100 which Urist reported in rabbits. This high score in rabbits in comparison with other animal species was attributed by Urist to the high degree of stability of the BMP and the species sensitivity of the rabbit for induction (Urist & Craven 1970).
The chronologically described events in and around the implant in the untreated animals (resorption of the implant, induction of bone tissue and mineralization and remodelling of this tissue) correspond with the course of events described by Urist and his co-workers (Urist et al. 1967; Urist et al. 1969). In our model, too, formation of new bone was observed in the areas in which the implant resorption had started. Urist regarded this resorption as a conditio sine qua non for positive bone induction (Urist et al. 1967; Urist 1970b). In addition he observed a relationship between the number of osteoclasts present and the degree of induction: 'the larger the number of multinucleated cells, the greater the yield of new bone that was obtained from an implant (Urist & Craven 1970).
One of our striking findings, however, was that cartilage induction was often observed in the areas in which no implant resorption occurred and in the pre-existent spaces of the implant. A possible explanation of this finding can be found in the study published by Ham & Harris (1972). They established that a low local oxygen tension in callus tissue stimulates the differentiation of the osteogenetic cells to chondroblasts. The same conclusion could be drawn from the experiment described by Van de Sandt (1977), who observed production of cartilaginous tissue after local destruction of the periosteal vascularization. This will result locally in a disturbance of oxygen tension.

Further arguments can be derived from in-vitro experiments with decalcified implants. It appeared that cartilage induction was established at a low oxygen tension, while administration of a sufficient amount of oxygen led to the formation of induced bone tissue (Nogami & Urist 1970; Nogami & Urist 1974). It seems likely, therefore, that local oxygen tension is one of the main determinants of differentiation of the osteogenetic cell to osteoblasts or to chondroablasts in the in-vivo induction process as well.

We also observed that capillaries normally grow into the implant spaces formed as a result of resorption Consequently there is a causal relation between the degree of resorption and the degree of vascularization (and therefore oxygen tension) in the implant Disturbed implant resorption, therefore, should lead to a marked increase in the formation of induced cartilage Support for this assumption could be derived from the experiments of Koskinen et al (1972), Simmons et al (1974) and Thompson & Urist (1970) Homologous implants, as used in our experiments also, normally provoke an adequate 'inflammatory reaction' mainly due to the antigenicity of the implant (Bang 1972) According to Buring (1975) this reaction comprises the production of multinucleated giant cells and osteoclasts from mononuclear cells. If this reaction is suppressed to a great extent by means of cortisone, then resorption diminishes and cartilage induction becomes predominant The assumption that a relation should exist between bone induction and vascularization is corroborated by the finding that the formation of marrow tissue is associated with a gradual replacement of cartilage by bone. Following this resorption, contact is established between the inductor in the implant (BMP) and the mesenchymal cell to be induced. The mechanism by which BMP transmits its signal to this mesenchymal cell is still obscure, but an intensive contact between the implant and the tissue, containing osteogenetically competent mesenchymal cells, seems necessary (Nogami & Urist 1974, Urist 1970b). An indication of the importance of this intensive contact in induction can be derived from the negative results obtained with infected implants in our series Comparable findings were reported by Urist (1970b; 1973). Apart from a possible lysis which can destroy the BMP, the inflammatory tissue causes encapsulation which precludes any contact between BMP and the cell to be induced. According to this reasoning, the lack of bone inducing properties of heterologous implants can partly be attributed to the encapsulation of these grafts (Bang 1972; Urist et al. 1967)

As a result of the induction, marked proliferation of osteoid and young bone tissue with an occasional islet of chondral tissue is observed during the third and the fourth week. It is often difficult to differentiate between mineralized and non-mineralized tissue in young bone in paraffin sections. An important indication for mineralization, however, can be the presence of a

'calcium trace' in the H-E preparations and the osteoid zones identifiable by its affinity for Alcian blue (Van den Hooff et al. 1966). Definite proof of the presence of mineralized tissue is supplied during the third week by microradiograms and the presence of oxytetracycline spots

Intensive contact between implant and newly formed tissue always exists. However, remineralization of the implant, which Linden (1975) considered to be necessary for an adequate course of induction, was not observed by the techniques we used According to these observations and the findings reported by Firschein & Urist (1972), Urist (1970b) and Urist et al. (1970), who found no electron-microscopic evidence of remineralization, it is highly improbable that remineralization of the implant occurs.

The results of our electron-microscopic study of untreated implants are consistent with the observations reported by Firschein & Urist (1972), Urist (1970b) and Urist et al (1970). These findings, and the further development of the bone formed by induction, warrant the conclusion that we are dealing with a normal de novo bone formation process, producing bone with characteristics similar to those of woven bone Unmistakable remodelling was found, with formation of centripetal haemopoietic bone marrow and centrifugal lamellar bone (Urist 1970a).

The high remodelling activity is also expressed in the diminished presence of the oxytetracycline label after the third week The Alizarin-complexon given during the fifth week, manifesting itself in more intensive lines, reflects the lamellar apposition The ultimate result of the induction process after six weeks is an oval-shaped, mature ossicle containing fully developed bone marrow. The invariably observed oval shape is probably determined by the forces exerted by the surrounding muscular tissue to which the ossicle is subject, as suggested by Bridges & Pritchard (1958) and Chalmers & Ray (1962). As in bone which is part of the normal supportive apparatus, the surrounding muscle tissue inserts in the capsule formed around the ossicle Since we are dealing with an ectopic bone fragment which has no function in the supportive apparatus, it is interesting to establish whether the ossicle formed has any survival chances. This is answered by the observation of Urist et al. (1967). They reported that the ossicle formed after homologous implantation in the abdominal muscles of the rabbit, was still intact after 1 year Our findings however, showed a slight regression in bone and marrow tissue after 10 - 12 weeks, and this might indicate a slow disappearance

The obtained results have made it clear that the behaviour of the induction process discussed shows some rather marked changes in response to EHDP-treatment In addition to diminished implant resorption, we observed large amounts of osteoid-like tissue and extensive chondroid areas. The latter

appeared to diminish in size during the fifth and the sixth week after implantation under continuous EHDP-administration. No indications of mineralization of any significance were found Centrifugal remodelling with formation of lamellar bone did not occur under continuous EHDP-treatment, and no ossicle was consequently formed

Although many data on the possible effects of EHDP on the behaviour of pre-existing bone have been reported, the literature on the effects of EHDP on an osteoneogenesis, e g. Urist's induction model, is very scanty Only Strates and Chalmers described experiments which can be compared with our experimental set-up Strates et al. (1971) failed to show radiologically mineralization in implants in rats treated with EHDP (15 mg/kg/day, subcutaneously); however, no histological study was made. More recently, Chalmers et al. (1975) described that a daily intramuscular dose of 2 5 mg/kg caused marked inhibition of bone induction in rabbits; in their publication, too, the histological data are not described in detail

On the basis of our experimental data, described in the previous chapter, an attempt will be made to answer the question concerning the possible sites of EHDP-activity. This will be done in a discussion of, successively, the possible influence of EHDP on implant resorption, on induction of bone tissue and on its mineralization and remodelling

Unmistakable diminished implant resorption was observed in the treated animals The number of osteoclasts had also markedly decreased. This agrees with the findings of Rosenblum et al (1976), in studying vital bone in situ in the proximal tibia of the rabbit Although they, too, failed to make an exact quantitative count, they reached the conclusion that the number of osteoclasts diminishes significantly in response to EHDP Substantial support for this observation can be derived from various reports EHDP has been shown to block parathyroid hormone (PTH)-stimulated resorption in tissue cultures. Several investigators confirmed this by determination of the ^{45}Ca released as a result of resorption (Fleisch et al 1968b; Fleisch et al.1969; Reynolds et al. 1972; Russell et al. 1970b) In addition EHDP was shown to prevent the PTH-induced increase in serum calcium in rats (Fleisch et al 1969) and reduces the bone turnover in rats (Gasser et al 1972) It was established on the basis of electron-microscopic findings that osteoclasts became inactive in response to EHDP – an inactivation manifested by shrinkage of the ruffled border (Schenk 1974) The multinucleated, vacuolated giant cells observed in our sections, sometimes in conglomerates, are difficult to classify. They may be 'frustrated' osteoclasts which are unable to fulfill their function (Van den Hooff 1976). This finding was reported in roughly similar terms by Schenk et al. (1973). One of the functions of PTH is stimulation of osteoclast production and activation of resting osteoclasts

(Parfitt 1976b) It is assumed (but by no means proven) that the sensitivity of the osteoclasts to PTH is reduced with EHDP. Rowe & Hausmann (1976), however, studied the effect of EHDP on osteoclasts in a tissue culture and concluded in favour of a direct toxic effect of EHDP on these cells. Although we cannot distinguish between these two possible effects, our observations clearly demonstrate a diminished implant resorption.

The formation of tissue, however, was not inhibited. This contradicts the description given by Chalmers et al. (1975). They stated that EHDP inhibited induction in rabbits.

The tissue formed, however, lacks the normal light-microscopic features of osteoid, while a predominance of chondroid structures was found during the first weeks

Since no quantitative determinations were made in our study, we are unable to answer the question whether EHDP has a stimulating effect on the amount of osteoid-like tissue formed. In response to parenteral EHDP-doses, as used in our experiments, a distinct increase in non-mineralized osteoid is generally observed in the pre-existent bone (King et al. 1971). The majority of investigators ascribe this increase entirely to the blocking effect of EHDP on the mineralization of the osteoid formed (Russell et al. 1973; Schenk et al. 1973). Substantial support can be derived from quantitative studies performed by Russell et al. (1973) Therefore an increased production of osteoid in response to EHDP can be regarded as improbable.

Concerning the structure of the tissue formed in response to EHDP, we chose the peculiar term 'osteoid-like' in order to indicate that we are dealing with atypical osteoid: poor in cells and fibres and presenting an 'aqueous' aspect. An additional striking feature was the presence of extensive chondroid structures

The atypical osteoid tissue observed shows similarities to the type of woven bone formed in response to EHDP in Paget's disease (Te Velde & Bijvoet 1976) It is to be noted that none of the many publications reporting an increase in osteoid in response to EHDP mentions this atypical aspect of the osteoid. This applies both to the studies on patients (Bijvoet et al. 1974), including ectopic ossifications (Smith et al. 1976), and to animal experiments (King et al 1971; Rosenblum et al. 1976; Russell et al. 1973; Schenk et al. 1973). It is to be borne in mind, however, that the observations described in in-vivo experiments with EHDP all concern pre-existent bone tissue, particularly metaphyseal and diaphyseal bone tissue. This is in contrast with the de novo bone involved in our experiments.

The presence of extensive chondroid areas might at least partly be explained on the basis of the disturbed resorption of the implant, resulting in a diminished vascularization and consequently a low oxygen tension The decrease of

the amount of chondroid tissue parallelling the formation of the highly vascularized marrow, agrees with this assumption. The absence of cartilage from the well-vascularized woven bone in Paget's disease in response to EHDP confirmes also this reasoning (Te Velde & Bijvoet 1976). There is a disagreement between our findings and the results obtained by Schenk et al. (1973). They described a persisting of hypertrophic chondrocytes in well vascularized rat epiphyses during continuous EHDP-exposure.

In our experiments no tendency of the osteoid-like tissue to decrease in volume within 6 weeks was observed. In the literature this phenomenon is reported as having been observed after a much longer period of EHDP-administration (12 weeks), both in patients (Bijvoet et al. 1974) and in dogs (Cabanela & Jowsey 1974).

More detailed examination of the interstitial substance of the osteoid tissue showed that the collagen had abnormal features. The electron-microscopic observations showed that the collagen fibrillae localized in the vicinity of the cells were often irregular and fragmented with frayed ends. Similar features are found in the woven bone in Paget's disease during EHDP-administration (Te Velde & Bijvoet 1976). With regard to osteoid formed in pre-existent bone in response to EHDP, however, it is explicitly noted that the collagen structures in this osteoid are normal (Rosenblum et al. 1976). An interpretation of the abnormal features found in the newly formed bone cannot be given in the scope of this study.

Another remarkable finding was the alcianophilia of the osteoid-like tissue indicating a high concentration of acid mucopolysaccharides in the interstitial substance. This is in agreement with the description given by Larsson & Larsson (1976) and with the histochemical findings reported by Rosenblum et al. (1976). It was noted in chapter 1 that hydrolysis of acid mucopolysaccharides, giving rise to the release of calcium, is important in starting mineralization. Larsson & Larsson (1976) suggested that this hydrolysis is blocked by EHDP and therefore no mineralization should occur.

Apart from this abnormal structure of the matrix, the cells are also pathological. After 3 - 4 weeks of EHDP-administration the osteoid-like tissue was found to contain cells resembling both osteoblasts and osteocytes. For lack of a better designation we call these 'atypical osteocytes' (Van den Hooff 1976).

Although descriptions of cell features in response to EHDP are scanty, hypertrophy of the chondrocytes was observed during EHDP-treatment in vitro (Bisaz et al. 1973) as well as in vivo (Larsson & Larsson 1976). Rosenblum et al. (1976) also reported that the cells in the osteoid during EHDP-treatment in pre-existent bone show a microscopic resemblance to osteoblasts as well as to osteocytes. Electron-microscopic examination of these

'atypical osteocytes' revealed signs suggestive of hyperactivity, e.g. extensive RER and pronounced Golgi areas. The obtained data do not allow to conclude on the existence of a relationship between these features and the pathological structure of the matrix.

In summary, it can be concluded that the osteoid-like tissue obtained by induction, during exposure to EHDP, presents atypical cellular as well as intercellular features

Apart from the possible interference of EHDP with matrix formation and matrix composition, the wellknown inhibitory effect of EHDP on mineralization in this matrix is to be considered. In our experimental set-up, too, no detectable mineralization was demonstrable with the used techniques during continuous EHDP-administration (5 mg/kg/day, intramuscularly) This block of mineralization is consistent with data in the literature concerning the effect of EHDP on pre-existing bone as well as on osteoneogenesis (Strates et al 1971)

Beside the above mentioned possible blocking of the mineralization by the inhibition of EHDP on the hydrolysis of acid mucopolysaccharides, several other theories on the inhibitory effect of EHDP on this process has been proposed It is widely accepted that EHDP prevents the conversion of amorphous calcium phosphate compounds to crystalline structures (Francis 1969) With regard to the mechanism of action of EHDP it is hypothetically possible that EHDP occupies the position of the biological pyrophosphate (PP_i) on and in the microcrystals. Unlike PP_i, however, EHDP is not hydrolysed, and this means that the microcrystal cannot continue its growth until the EHDP has been washed out of it. A more rational alternative theory on the action of EHDP is that of a competitive inhibition of the activity of the enzyme pyrophosphatase by EHDP (Woltgens 1974; Woltgens et al. 1971; Woltgens et al. 1973). This inhibition precludes degradation of the pyrophosphate and in this way the growth of the microcrystal is prevented. Our electron-microscopic findings in pre-existent calvarial bone were consistent with the suggested inhibitory effect of EHDP on microcrystal growth. Only small, circumscribed densifications were found in the non-mineralized tissue; at analysis (EDAX) these densifications showed a distinct calcium peak These deposits, however, were absent in the newly formed bone tissue in the EHDP-treated implants and screening of the non-mineralized tissue by EDAX-analysis failed to reveal calcium accumulations. From these data it might be a logical but unverified conclusion that EHDP interferes with mineralization also before the formation of amorphous calcium phosphate compounds or microcrystalline structures. The absence of calcium deposits may imply that release of calcium by hydrolysis of the acid mucopolysaccharides is blocked by EHDP, as assumed by Larsson & Larsson (1976). The

alternative possibility is that EHDP interferes with the cellular metabolism and consequently with the release of calcium from the cell to the matrix. An argument in favour of such an effect, although of a transient nature, can be derived from the electron-microscopic observations revealing in the calvaria of the treated animals, an excessive storage of calcium in the mitochondria of the osteoblasts after 1 week of EHDP-administration. After 2 weeks of EHDP-administration, however, this accumulation had definitely diminished. In the EHDP-literature no comparable data on in-vivo experiments are reported. In an in-vitro experiment with rat kidney cells, however, Guilland et al. (1974) demonstrated transient accumulation of calcium in the mitochondria in response to EHDP. Their experiment showed that the duration of this transient EHDP-effect could be reduced by adding parathyroid hormone to the medium. Since PTH normally stimulates the release of calcium from the mitochondria (Parfitt 1976b) an interference of EHDP with the course of events leading to a PTH-stimulated Ca^{++}-release might be possible.

In summary it can be stated that an induced osteoid formation during continuous EHDP-administration, not only results in an abnormal matrix but also in a lack of mineralization of this matrix. The obtained data suggest the existence of two different effects of EHDP. First prevention of the growth of microcrystals by chemisorption and second by an effect of EHDP on the release of calcium from the mitochondria to the matrix.

The non-mineralization of the osteoid-like areas has further consequences. In our sections of treated implants, a persistence and a substantial increase of the osteoid-like tissue was observed without clear signs of remodelling, which was manifested by the presence of only a small number of osteoclasts in the formed tissue. A similar finding was reported by Rosenblum et al. (1976) who studied the influence of EHDP on pre-existent bone. They suggested that the presence of minerals is a prerequisite to, or possibly a signal for, osteoclast activity. As our experiments showed, however, this suggestion does not apply for demineralized implants. A more plausible interpretation, therefore, is that proposed by Parfitt (1976a). He maintained that a matrix, once mineralized, shows a structure which is recognizable for the osteoclasts. Since the osteoid-like tissue, formed during EHDP-administration, seems to lack any detectable mineralization, remodelling is bound to lag behind and this might explain the persistence of the osteoid-like tissue.

An alternative, although more hypothetical possibility, which might account for the abundant presence of osteoid-like tissue can be derived from the experiments of Dubuc & Urist (1967). They demonstrated that adequate demineralization is a prerequisite for optimal functioning of the induction mechanism. Moreover, the matrix obtained by induction can also function as

inductor as long as it is not or only partly mineralized (Urist et al. 1967). According to these findings the unmineralized matrix formed in response to EHDP, can function as a second inductor. This may result in increased production of osteoid and might at least partly explain the difference between the amount of osteoid in control and EHDP-treated implants.
From the experiments in which EHDP-administration was discontinued, it appeared that the duration of the recovery process is highly dependent on the exposure time. It appeared that a small but significant effect was observed when the animal was treated during the first week after implantation. The deleterious effect was still visible 5 weeks later. Although a marked similarity in the gross structure was found as compared to the untreated implants, the ossicle still contained immature bone tissue and showed a more trabecular structure.
A recovery phase of only 3 weeks after 3 weeks of EHDP-administration was evidently less adequate: slight mineralization was observed in only a few implants, while the structure of the osteoid-like tissue was highly similar to that of the implants treated for 6 weeks. Although after 4 weeks of EHDP-administration, a distinct increase in mineralized tissue was found after successive survivals of 8, 10 and 12 weeks, the presence of a large amount of trabecular bone tissue in the 12 weeks survivals clearly demonstrates that a complete recovery lasts much longer. The observed slow restoration of the mineralization after the withdrawal of EHDP as well as the persistence of the osteoid-like tissue is highly likely due to a slow disappearance of the EHDP from the tissue. The disappearance of the EHDP from pre-existent bone has been described by King et al. (1971) as a kind of diffusion process: 'Bleeding of EHDP from the osteoid and calcified borders would permit calcification to start again'.

Although the structure of periarticular ossifications in the initial phase after total hip replacement shows a close resemblance to the induced bone tissue (Nollen & Slooff 1973), our experimental findings can only be translated into clinical terms with reservations. The use of EHDP in prevention of periarticular ossifications shows comparable effects with the inhibition of mineralization in our experimental model. A difference, however, was found in the recovery-time after discontinuation of EHDP-administration. Bijvoet et al. (1974) reported that, 2 weeks after discontinuation of oral EHDP-administration (20 mg/kg/day), periarticular ossifications were again radiologically demonstrable in treated patients. This could perhaps be explained on the basis of the mode of administration. Michaël et al. (1972) found that the half-life of EHDP in dogs was 12 days after oral administration. This is significantly shorter than the calculated 30 days after parenteral administra-

tion in rats (King et al. 1971). Michaël et al. (1972) suggested that, after parenteral administration, more marked diffusion of EHDP in the bone occurs as a result of the temporarily higher serum EHDP-concentration.
Radiologically no evident difference in periarticular ossifications was found between controls and treated patients some 3 months after discontinuation of EHDP-treatment (Bijvoet et al. 1974). Although radiological data give an general impression of the degree of mineralization, histological studies are necessary to get more detailed information about the structure of the treated tissue. The translation of our experimental findings into clinical terms should imply, however, that after EHDP wash-out, the periarticular ossifications in treated patients will ultimately not be different in size and structure from those in untreated patients. In that case the only remaining clinical benefit is the fact that preventive temporary EHDP-treatment ensures increased hip mobility and diminished pain sensations if periarticular ossifications occur after total hip replacement (Bijvoet et al. 1974).

SUMMARY AND CONCLUSIONS

This study was motivated by clinical trials with EHDP to prevent periarticular ossifications after total hip replacements. The need was felt for further evaluation of the influence of EHDP on ectopic bone tissue. For this purpose, use was made of the induction model evolved by Urist and his co-workers. There is a certain similarity between ectopic bone formation obtained in this way, and the bone found in the initial phase of periarticular ossification. Besides a comprehensive histological study, an electron-microscopic study was made of the induction model and of calvarial bones in an effort to establish possible cellular changes in osteoblasts and osteocytes in response to EHDP.
By way of introduction to the problem statement, a brief review of the literature on bone formation, with special reference to mineralization, induction of heterotopic bone and the effect of diphosphonates is presented.
The results obtained would seem to warrant the following conclusions and considerations.

1. The induction model developed by Urist proved to be a suitable model par excellence for testing the effect of chemical substances on bone tissue.
2. EHDP has no inhibitory effect on the induction itself, but the features of this tissue obtained differ fundamentally from those in untreated animals.

3 The presence of large areas of chondroid structures is probably to be ascribed to a disturbance of implant resorption caused by EHDP. The consequently diminished vascularization favours differentiation of the osteogenetic cells to chondroblasts
4. The cells and interstitial substance in the osteoid formed in response to EHDP, show qualitative differences from those in untreated implants.
5 The tissue formed in response to EHDP shows hardly any remodelling. The extent of the chondroid structures diminishes when vascularization improves.
6. No mineralization of the induced tissue occurs during continuous EHDP-administration.
7. After discontinuation of EHDP-treatment, a recovery phase can be expected, tending to the formation of an normal ossicle.
8. The existence of either a direct or an indirect interference of EHDP with the cellular metabolism is among the real possibilities.
9. The similarity in structure between the bone obtained by induction in our experiment and that found in the initial phase of periarticular ossification, does not warrant the conclusion that these ossifications result from a comparable induction process.
10. The clinical translation of our experimental data warrants, with reservations, the following prediction The periarticular ossifications ultimately found after discontinuation of EHDP-treatment in patients after total hip replacement, will not differ from those in untreated patients.

SAMENVATTING EN CONCLUSIES

De motivatie voor ons onderzoek is gelegen in de kliniek, waar klinisch experimenteel EHDP werd toegediend ter preventie van periarticulaire ossificaties na totale heupvervangingen. Er bestond behoefte aan een nadere evaluatie van de inwerking van het EHDP op ectopisch gelegen botweefsel Hiertoe werd gebruik gemaakt van het door Urist en zijn medewerkers ontwikkelde inductiemodel. Er bestaat een sterke gelijkenis tussen het aldus verkregen ectopische bot en het periarticulair aangetroffen bot in de beginfase. Naast een histologische studie werd er een electronenmicroscopisch onderzoek verricht op het inductiemodel en op calvaria naar mogelijke cellulaire veranderingen van de osteoblast en osteocyt onder inwerking van het EHDP.
Ter introductie op de problematiek werd een korte verhandeling gewijd aan de botvorming met het accent op de mineralisatie hiervan, de inductie van heterotoop bot en de werking van diphosphonaten.

De verkregen resultaten geven aanleiding tot de volgende conclusies en overwegingen.

1. Het door Urist ontwikkelde inductiemodel voldeed aan alle verwachtingen en blijkt bij uitstek geschikt te zijn voor het uittesten van de inwerking van chemische stoffen op botweefsel.
2. Van een remming van het inductieproces zelf door het EHDP is geen sprake. De aard van het verkregen weefsel verschilt echter essentieel van dat bij de onbehandelde dieren.
3. De aanwezigheid van grote velden kraakbeenachtige structuren is waarschijnlijk terug te voeren op de door het EHDP verstoorde resorptie van het implantaat. De daardoor verminderde vasculaire voorziening bevordert een differentiatie van de voorlopercel in chondroblasten.
4. De cellen en de tussenstof in het onder inwerking van het EHDP gevormde osteoid, vertonen kwalitatieve verschillen t.o.v. de onbehandelde implantaten.
5. Een remodellering van het onder inwerking van het EHDP gevormde weefsel wordt amper gezien. Bij een verbetering van de vascularisatie neemt de omvang van de kraakbeenachtige structuren af.
6. Bij voortdurende toediening van het EHDP treedt geen mineralisatie van het geinduceerde weefsel op.
7. Na het staken van de EHDP-toediening is een herstelfase te verwachten, neigend tot de vorming van een normaal ossiculum.
8. Het bestaan van een directe danwel indirecte inwerking van het EHDP op het cellulair metabolisme behoort tot de reële mogelijkheden.
9. Uit de gelijkenis tussen de structuur van het via inductie verkregen bot in ons experiment en de periarticulaire ossificaties in het beginstadium, mag niet ipso facto geconcludeerd worden dat deze ossificaties een gevolg zijn van een vergelijkbaar inductieproces.
10. Bij een vertaling van onze dierexperimentele gegevens naar de kliniek kan onder voorbehoud de volgende verwachting uitgesproken worden: de uiteindelijk, na het staken van de EDHP-toediening, optredende periarticulaire ossificaties na een totale heupvervanging, zullen niet verschillen van die bij de onbehandelde patienten.

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STELLINGEN

I

Het is wenselijk het gebruik van diphosphonaten ter preventie van ectopische ossificaties bij een totale heupvervanging vooralsnog als experimenteel te beschouwen.

II

Een interferentie van disodium ethane-1-hydroxy-1,1-diphosphonaat (EHDP) op het cellulaire botmetabolisme is alleszins waarschijnlijk.

III

Het onderzoek naar het aanwenden van menselijk homoloog bot mag niet beperkt blijven tot de huidige botbankprocedure.

IV

De dwarstractie met stabilisatie is een belangrijke aanvulling van het Harrington-instrumentarium bij de behandeling van scoliosis.

V

Stuitpijnen kunnen het gevolg zijn van een aandoening in het lumbosacrale gebied. Het verdient dan ook aanbeveling om bij hardnekkige stuitpijnen naast het maken van sacrumfoto's een neurologisch onderzoek, lumbaal punctie en myelografie te verrichten.

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VI

Bij de behandeling van patiënten met chronische rheumatoide arthritis behoort de orthopaedisch chirurg reeds in een vroeg stadium zijn inbreng te hebben.

VII

De grote vlucht van de orthopaedische operatieve mogelijkheden maakt de uitspraak: „J'ai une idée, pauvres malades" weer zeer actueel.

Prof. Dr. G. M. San Giorgi, 1910-1969

VIII

De eerste geslaagde bottransplantatie en tevens voorwaarde zonder meer tot het verrichten van alle daarop volgende transplantaties, wordt aangegeven in Genesis hoofdstuk 2, vers 23, met de zinsnede: „dit is nu eindelijk bot van mijn botten".

IX

De circulaire doorsnijding van het periosteum heeft klinisch geen betekenis voor het opheffen van beenlengteverschillen.

H. M. van de Sandt. The influence of transverse section of the periosteum on the growth of the rabbit femur. Diss. Nijmegen (1977).

X

Hoewel de postnatale screening op Congenitale Hypothyreoïdie (CHT) absoluut noodzakelijk is, moet men zich realiseren dat op een later moment een CHT alsnog manifest kan worden.

G. A. De Jonge, T. Kindergeneesk. 45, no. 1, 1977.

XI

Het grote aantal postoperatieve trombosen in de orthopaedische chirurgie, zoals kan worden vastgesteld met J^{125}-fibrinogeen en/of phlebografie, behoort voor de orthopaedisch chirurg reden te zijn de nodige aandacht te schenken aan prophylaxe in deze.

XII

Subcutane, lage doses heparine als methode tot tromboseprophylaxe in de orthopaedische chirurgie moet zijn waarde nog bewijzen.

XIII

De op de consument gerichte gezondheidszorg kan worden verbeterd door paramedisch personeel op te leiden tot het geven van adviezen.

B. Rushmer, Group Health Care Seattle, 1976.

XIV

Het wetsontwerp „Voorzieningen Gezondheidszorg" geeft in feite aan de overheid een blanco volmacht om zonder de gebruikelijke parlementaire controle het particuliere initiatief in deze sector te doden.

XV

De belangrijke bijdrage van een co-referent in een promotiestudie laat zich niet raden uit zijn staanplaats tijdens de promotie.